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1986 MEETING OF THE AMERICAS BRANCH OF THE ELECTROPHORESIS SOCIETY

MARCH 26-28, 1986

PROCEEDINGS

Editor: Dennis J. Reeder

U.S. DEPARTMENT OF COMMERCE
National Bureau of Standards
National Measurement Laboratory
Center for Analytical Chemistry
Gaithersburg, MD 20899

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NATIONAL BUREAU OF STANDARDS
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THE ELECTROPHORESIS SOCIETY

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INTRODUCTION

The Electrophoresis Society (America's Branch) in 1985 had the task of deciding whether to hold a national meeting in 1986, the same year as the planned international meeting in London. As the council met, the consensus was that there would be sufficient interest in a meeting that plans should be made. The authors of the plenary sessions were contacted and graciously committed to the task of writing papers for publication in the proceedings.

The meeting format was agreed upon to allow maximum interactions among the participants, to allow sufficient time for plenary lecturers to give a comprehensive lecture, and to enable the attendees to have a published document for later reference.

The afternoon meetings were structured to permit the greatest information exchange by arranging sessions on selected topics, moderated by session chairpersons who would facilitate the direction of the presentations. The cooperation and willingness of individuals to participate would be the key to a successful information exchange. The informal nature of the sessions was devised to allow the expert and the novice to exchange findings and details about their research.

The impact of electrophoretic separations on biochemical analyses is reflected in the topics covered in this publication. Two-dimensional electrophoresis (2-DE) has matured to become an accepted tool for clinical studies. Immobilized pH gradients are becoming more reproducible and have found use as the first dimension high resolution separation step in 2-DE. The chemistry of protein detection in gels has progressed significantly to enable quantification. Although not fully understood, studies of staining mechanisms are proceeding. Nucleic acid separations in gels is a common technique, but the theoretical aspects of the separation process are just now being explored. Standardization in electrophoretic separations is addressed in relation to new materials for molecular weight, size and charge standards. The beginnings of interlaboratory standardization by round-robin studies also is reported. New matrix materials for separations are introduced . Finally, the practical applications of many techniques are reported.

ACKNOWLEDGEMENTS

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DISCLAIMER

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PLENARY PAPERS

MAKING ULTRATHIN-LAYER POLYACRYLAMIDE GEL ISOELECTRIC FOCUSING A REPRODUCIBLE METHODOLOGY

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Introduction

Recent advances in isoelectric focusing have shown that proteins can be separated on ultrathin-layer polyacrylamide gels ($\leq$ 0.36 mm thick gels) (1). The advantages of ultrathin-layer polyacrylamide gel isoelectric focusing (ULPAGIF) are increased resolution and sensitivity of detection, shorter separation and staining times, and reduced cost. Ultrathin-layer gels have a greater surface area-to-volume ratio than thicker gels and, therefore, are more effective at heat dissipation. Thus, higher voltages can be applied to the gels and an increased concentration of synthetic carrier ampholytes (4%, w/v) can be incorporated into the gel with the expectation of superior resolution. Furthermore, the higher voltage gradient produces narrower protein bands (2, 3), which results in more protein per unit gel volume. Since this, in effect, presents more protein for the subsequent assay, the sensitivity of detection of the system is increased. The applied higher field strengths also result in faster separation times making it possible to type more samples in less time than previously possible. Budowle (3-9) has shown that the separation times by ULPAGIF for the genetic markers phosphoglucomutase-1 (PGM), erythrocyte acid phosphatase

(EAP), alpha 1-antitrypsin (Pi), group-specific component (Gc), transferrin (Tf), esterase D (EsD) and hemoglobin (Hb) can be as fast as 37, 22, 75, 125, 135, 53 and 25 minutes, respectively. Due to shorter diffusion pathways, assay times [such as for silver staining (10)] are greatly reduced. Rapid staining is an important consideration particularly for functional assays for polymorphic enzymes where diffusion of the isozyme patterns can reduce resolution. Finally, by reducing the gel thickness, the quantities of reagents (and thus the cost) are reduced without compromising the number of samples analyzed.

Despite the advantages of using ultrathin-layer gels, there has been a reluctance to routinely use this methodology for genetic marker typing. Part of this reluctance can be attributed to a lack of reproducibility of banding patterns from gel to gel and lack of linearity across a gel. Problems with variation in resolution and wavy patterns can lead to possible mistypings, increased inconclusive determinations and time-consuming re-typing of samples. These problems are surmountable provided certain considerations are incorporated into ULPAGIF protocols.

A). Casting Techniques

Care should be exercised in the manner the gels are cast. There are two general methods for casting ultrathin-layer polyacrylamide gels - capillary and flap techniques [see Allen (11) and Radola (12) for the methodology]. After gels are poured using the flap technique, a weight is placed on the top glass plate to seal the gel. If this weight is not evenly distributed, the resulting gel will not have a uniform thickness. The results

are distorted protein patterns and undesired gel drying. Therefore, several glass plates should be used to evenly distribute the weight (4).

The capillary method in comparison to the flap technique is less time-consuming and allows for easier gel preparation. The major problem with the capillary method is avoiding the production of bubbles in the gel during pouring. This problem can be solved by lightly tapping the plate at the gel solution front as it is migrating under the plate by capillary action. This will produce an even flow of the gel solution and eliminate bubble formation. In the event that a bubble is trapped under the glass plate, simply slide the glass plate to expose the bubble, the bubble will dissipate, and then slide the plate back to its original position. After the gel polymerizes remove it from the casting tray and wrap it in plastic wrap. Gels can be maintained in the refrigerator in this condition from one to three weeks. In contrast, gels cast by the flap technique can be maintained at room temperature from three to six months (unpublished data).

The methods developed by Budowle (3-9) routinely used 200 um thick gels instead of thinner gels used by others (12-18). Even with the best of care in casting gels, there are still some slight variations in thickness within a gel. A 10% decrease in a portion of a 200 um thick gel (20 um) is less deleterious than a 10% decrease in a portion of a 100 um thick gel (10 um). The latter gel, at times, yielded uneven migration of bands, resulted

in protein migrating along the gel surface instead of entering the gel and/or dried out (unpublished data and personal communication, R. C. Allen).

B). Effect of Electrode Distance

As the distance between the electrodes is reduced the minor depressions in the gel present less of a problem (due to increased conductivity). Kinzkofer and Radola (19) have had great success with 50-100 um thick gels with inter-electrode wick distances of 1-3 cm. However, for the purposes of genetic marker typing these inter-electrode wick distances are impractical. The pH ranges of commercially available synthetic carrier ampholytes are not narrow enough for such distances and thus impose limitations on gel dimensions. Allelic products with slightly different isoelectric points lie too close together to resolve in this manner, especially when functional enzyme overlay assays are employed (diffusion problems). When the inter-electrode wick distance is 5-10 cm, as is necessary for most genetic marking typing systems, 200 um thick or thicker gels should be employed. Use of a gel with this thickness will contribute favorably to the reproducibility of the system.

C). Effects of Sample Protein and Salt Concentrations

Excess protein loading is tolerated more by thicker gels than thinner ones. For example, in PGM analysis, 6-8 ug of protein can be applied to 200 um thick gels with an 8 cm inter-electrode wick distance containing 4% (w/v) pH 5-7 ampholytes (LKB) and produce linear PGM subtype patterns (20, 21). Less than half that protein concentration can be applied to a similar

gel, 100 um thick, without producing bowed PGM subtype patterns. Similar trends occur for salt loading. However, in the case of salts, diluting the sample (and still applying the whole sample to the gel) can reduce deleterious salt effects. It appears the limiting factor is the amount of salt per unit sample volume and not the total amount of salt. The effect of salts led Pflug (21) to develop a wedge-shaped gel for PGM subtyping of semen samples. The gels were 300 um thick at the anode decreasing in thickness to 50 um at the cathode. Pflug applied semen stain extracts at the thicker portion of the gel. There was less distortion of PGM due to the salt effects. The wedge-shaped gel combined the higher resolving capacity of ultrathin-layer gels with the loading capacity of thicker gels. In contrast, Budowle, et al. (22), utilizing a straight 200 um thick gel, a reduced inter-electrode wick distance of 8 cm and a much higher final voltage gradient (290 V/cm vs. 180 V/cm), observed no deleterious salt effects on PGM from semen stains.

It should be noted that protein loading tolerances are also dependent upon the pH range and the particular manufacturer of the synthetic carrier ampholytes. For example, more than twice the protein that can be applied to a pH 5-7 ampholyte (LKB) gel can be applied to a pH 4.5-5.5 Servalyte gel without any protein band distortion. While a gel containing pH 4.5-5.4 Pharmalytes can only handle 70% of the protein of the Servalyte gel (unpublished data).

D). Effect of Field Strength

The primary advantage of ultrathin-layer polyacrylamide gels is the ability to use higher field strengths. Rilbe (23), Giddings and Dahlgren (24), and Allen (11) have shown that resolution is proportional to the square root of the voltage gradient. Since these gels can more effectively dissipate Joule heat, higher field strengths can be utilized with the expectation of increased resolution. For gels with an inter-electrode distance of 5-10 cm, voltage gradients as high as 300 - 700 V/cm have been used (2-12, 20, 22). Furthermore, the higher voltage gradient can produce narrower protein bands (2, 3). The concentrating of protein bands into more narrow zones results in more protein-per-unit gel volume which, in effect, presents more protein for the subsequent assay. Allen and Arnaud (2) using the Rohament P enzyme test demonstrated that a ten-fold voltage gradient increase (50- 500 V/cm) yielded a 2.3-fold increase in the number of bands resolved and a band-width decrease by a factor of 2.3 to 2.5. Budowle and Murch (3) observed that by increasing the voltage gradient from 340 V/cm to 460 V/cm a one-third decrease in the width of Pi bands could be realized. Many investigators (14-18) have utilized ULPAGIF, but do not take obvious advantage of the application of higher field strengths. Usually these investigators used voltage gradients less than 200 V/cm. Genetic variants with very close isoelectric points, such as the C1 and C3 allelic products of Tf (25-27), the M3 and M2

allelic products of Pi (28), and rare variants of Hb, can be easily resolved when higher field strengths are employed (300 V/cm - 700 V/cm).

A problem that manifests itself with the use of certain ampholyte ranges and high field strengths is the appearance of "hot spots" (29). These "hot spots" are conductivity gaps in the gradient and serve as a limiting factor for the field strength that may be applied to the gel. With sufficiently high field strengths the "hot spots" become burn spots on the gel (9, 11). This is an undesirable effect, especially when the protein(s) of interest has yet to be resolved. The problem can be solved by adjusting one of the electrodes (after a prescribed time into the run) to bypass the area of the gel where the conductivity gap exists. There then will be a shorter inter-electrode wick distance, a more uniform conductance across the gel, no gel burning, and the run can be completed to obtain the desired resolution. This approach has been successfully used for subtyping Gc in bloodstains (9).

E). Reproducing the Voltage Gradient

Not only is resolution dependent upon the voltage gradient, but for ultrathin-layer polyacrylamide gels, the voltage gradient is also important for gel-to-gel reproducibility and linearity across a gel. Budowle (7, 20) demonstrated that once the ideal voltage gradient conditions were empirically determined, the voltage gradient had to be reproduced at intervals for every ULPAGIF run. Although the gels were still

focused with constant power, the power mode was adjusted at regular intervals depending upon the voltage parameter. By following this approach, the same results were obtained from gel to gel. To produce band linearity across a gel, it was found that the initial voltage (regardless of the inter-electrode wick distance) applied to the gel during prefocusing could not exceed 250 volts. As long as the conditions were reproduced, well-resolved, highly reproducible patterns were obtained. Further, this approach appears to compensate for ambient temperature and humidity effects. Current can also be a limiting factor. However, the previously reported methods (7, 20) have maintained the current at low enough levels so as not to have an impact on gel reproducibility.

F). Reagents

Fresh stock solutions of recrystallized acrylamide should be considered for ULPAGIF. Chrambach, et al. (30) have shown that impurities, such as acrylic acid, will confer ion exchange properties to the gel. This can cause irreproducibility as well as artifacts between runs. Budowle (6) observed that lower grades of acrylamide appear to inhibit EsD activity. This is presumably analogous to the effects of impure acrylamide on mouse plasma esterases reported by Allen, et al. (31). Fresh stock solutions of acrylamide (less than one month old) should be used to avoid distortions originating in the anodal portion of the ultrathin-layer polyacrylamide gel. Older stock solutions cause distortion in the gel resulting in waviness across the gel

so that similar proteins in different sample lanes can not be compared effectively. In addition, when a functional assay is utilized to visualize the polymorphic enzymes, the gels should sit overnight prior to use. Freshly poured gels tend to inhibit enzymatic activity. This phenomenon was readily observed for EsD and in particular for EAP (5, 6). The activity of the C band of EAP was completely inhibited in polyacrylamide gels that polymerized for only one to three hours. Thus, using fresh recrystallized acrylamide stock solution and permitting polyacrylamide gels to sit overnight prior to use are imperative for minimizing mistypings of genetic marker systems.

If the waviness which originates at the anode still persists, it is suggested to employ alternative anolytes. The anolyte for pH 5-7 ampholyte (LKB) gels for successful subtyping of PGM is phosphoric acid. However, when phosphoric acid is used as the anolyte with narrow-range Pharmalytes, an anodal distortion occurs. By using saturated L-aspartic acid instead of phosphoric acid the distortion is greatly reduced (9). Also, some narrow-range ampholytes which yield good separation of genetic variants. (such as Pharmalyte pH 4.2-4.9 for Pi (3), pH 4.5-5.4 for Gc (9, 32), and pH 6.7-7.7 for Hb (8)) may still exhibit an inherent instability in the gradient. To alleviate this waviness enhancing these narrow range ampholytes with small amounts (1:10) of wider range ampholytes (pH 4-6, pH 4-6.5 or pH 3-10) is required (8, 9). In addition to yielding more linear

patterns across a gel, enhancing the pH gradient with wider pH range ampholytes does not appear to compromise the separation distances between allelic variant bands.

There is one constraint on this methodology which is out of the control of the laboratory. This is the batch-to-batch variation of ampholytes. To subtype Tf (7) pH 5-7 ampholytes (LKB) are used. While lots 48 and 50 produced the desired patterns demonstrated in the literature, it was impossible to resolve the C1C3 phenotype when lot 49 was used (unpublished data). These problems have been observed for Servalyte and Pharmalyte as well. To avoid these problems in the future ampholyte manufacturers will have to take responsibility for quality control.

Following the approaches described above, reliable, reproducible, and linear band patterns can be achieved using ULPAGIF for genetic marker typing as well as for most isoelectric focusing protein analyses. This is of particular concern for laboratories with heavy case loads. Time and expenses taken to rerun samples may be prohibitive. Further, when sample size is a limiting factor, as can be the case for forensic samples or supplies of extremely rare variants, multiple tests to obtain an analysis may prove to be impossible. If care is taken, seldom will a gel have to be rerun.

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Polyacrylamide Gel Electrofocusing in pH gradients formed by Carrier Ampholytes and by Immobiline: Pro's and Con's.

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Ever since the introduction of immobilized pH gradients (IPG) by Gasparic et al. (1), one has to make a decision in the laboratory practice of "charge fractionation" whether to apply electrofocusing in IPGs (IPGEF) or whether to continue the isoelectric focusing technique in a pH gradient naturally formed by the carrier ampholytes (CA), designated here as IEF. The answer to that question will depend on the particular application and relevance of some of the following criteria: 1) Resolving power; 2) speed; 3) reproducibility; 4) volume, ionic strength and entry of the sample; 5) experimental simplicity; 6) ease of selection of the range and shape of the pH gradient; 7) susceptibility to pH and voltage measurement; 8) perturbation by gel hydration and dehydration phenomena; 9) artefacts; 10) compatibility with SDS-PAGE for 2D-separations; 11) ease of protein isolation, preparative load capacity and purity of

product; 12) equipment needs and cost.

Application of these criteria to IEF and to IPG electrofocusing (IPGEF) at this time must rest, unfortunately, on an extremely limited data base as far as IPGEF is concerned, compared with the many thousands of papers on IEF in the literature. This imbalance necessarily decreases the reliability of our conclusions.

Finally, the philosophical point needs to be made that IPGEF, just because it is the newer method, must not therefore necessarily supersede IEF. We take it for an axiom that every method has its proper and optimal area of usefulness, and that no single method is applicable or useful for all separation problems. The real question is to find these areas of specific applicability, both for IEF and for IPGEF.

1) Resolving power:

The resolving power in electrofocusing is proportional to the degree of flatness of the pH gradient as well as to field strength [equ.138 of (2)]. Narrow pH ranges and therefore flat pH gradients are applicable as a device promoting resolving power in those analytical or preparative separation problems which comprise components with closely distributed pIs. When the mixture to be resolved comprises a wide spectrum of pIs, a steep pH gradient, or a flat pH gradient of great length is required. The latter is impractical because electrofocusing at high field strength over a long gel requires impracticably high voltages and unduly extended focusing time. An attempt to resolve this dilemma by carrying out electrofocusing at a 45° angle to the orientation of the pH gradient has failed so far (3).

In separation problems to which narrow pH ranges and flat pH gradients are applicable, both IEF and IPGEF seem capable of providing pH gradients of approximately 0.5 pH units per 10-20 cm gel length, although the methods used to achieve flat pH

gradients are different.

a) In IEF, where only limited narrow pI-range CAs are commercially available, one can create the desired flat pH gradients in at least one of three ways : i) By preparation, using electrofocusing or other charge fractionation methods, of a narrower from an available wider pI-range; ii) by introduction of "separators" with pIs in the region of the pH gradient which one desires to flatten (4); iii) by "constituent displacement" (5,6) defined as the electrophoretic removal of undesirable CAs by electrofocusing between large catholyte and anolyte reservoirs at the desired terminal pHs of the gradient. It should be noted, however, that pH gradient flattening by the latter method is slow so that the desired pH gradient may not be realized prior to the deterioration of the gradient through the (usually) cathodic drift.

b) IPGEF allows one to compute pH gradients of any degree of shallowness (7). In a recent example, an IPGEF gradient of 0.01 pH unit/cm was achieved (8). It should be kept in mind, however, that the computations in view of non-ideal conditions may not be realistically descriptive of the pH gradient obtained experimentally in a gel, although they should approximate it. With a few trial-and-error modifications, one should be able to set up pH gradients of any degree of flatness in IPGEF. The work involved in these modifications certainly appears less than that which is needed for pH gradient flattening in IEF by any of the 3 methods discussed above. In addition to the design of overall flat pH gradients, IPGEF provides a computational procedure for allowing the formation of flat regions within a steeper overall pH gradient (9). Since the method allows for full freedom in the choice and location of a flat region of the pH gradient, it presents a considerable advance over local pH gradient flattening by separators (see above) and the associated trial-and-error procedures in IEF. In summary, IPGEF appears as the superior way to achieve high resolving power by pH gradient flattening.

To obtain maximal resolution both in IEF and in IPGEF, voltage should be raised so as to achieve maximal field strength within the limits of Joule heat dissipation by the apparatus. The overall conductance across the pH gradient is less by a factor of at least 20 in IPGEF as compared to IEF and correspondingly higher voltages are applied to IPGEF. Applied voltages of up to 1000 V/cm have been reported for IPGEF (10), while only exceptionally well cooled and thin gels in IEF permit application of as much as 600 V/cm (11). Values of 20-40 V/cm appear representative for most applications of IEF. It should be kept in mind, however, that in view of the unevenness of the conductance profile, documented at least for the pH-range 4-10 in IPGEF (12) and in IEF (13), it is the field strength at the point at which the separated zones are located which defines resolution, not the overall voltage.

It must also be realized that it is the actual and not the computed field strength which governs resolution. At least in the wide pH-range of IPGEF, the predicted (7) and experimental (12) field strengths vary considerably. In the pH 4-10 IPGEF system, this variation may be 25-fold within a single pH gradient (12). Similar variations in field strength obtain in IEF (13,14). When CAs are introduced into IPGEF, the overall conductance across the pH gradient increases. Again, this increase may or may not affect the question whether the local field strength is higher in ICAPG-EF than in IEF, and thus whether resolution is higher.

In conclusion, the higher field strength predicted for IPGEF should provide higher resolution than obtainable in IEF. Whether this increase in resolution actually occurs, depends on the real, experimentally measurable, field strength. Increase of resolution by means of pH gradient flattening is available to both IEF and IPGEF but more extreme flattening is possible, and flattening is achieved more easily, in IPGEF than in IEF.

2) Speed:

To compare the speed of IPGEF and of IEF, it is necessary to determine the time required for identical proteins to reach the steady-state focused position in a similar pH gradient. Such determinations were made in our laboratory, where the same mixture of colored proteins was applied to pH 4-10 gradients formed in IEF, in IPGEF and in IPGEF in presence of CAs (ICAPG-EF) (12). In each case, Vh/L^2, where L = gel length, were approximately equal so that the comparison of time was based on equivalent voltages and gel length (15). The results show that the relative speeds of the 3 methods vary within not more than a factor of 2. Within that range, IEF is the fastest method, followed by ICAPG-EF. IPGEF is the slowest of the 3 methods of electrofocusing. This is due to the fact that in spite of the high voltage the low conductance regions in the IPG gel present bottlenecks to the transport of the protein (and salt) and thereby delay the attainment of the isoelectric positions.

In conclusion, the widely held prejudice that IEF is faster than IPGEF is not substantiated by the data. The advantage is marginal, and only appears considerable in view of the delayed migration, due to electric field perturbations, in the horizontal slab technique of IPGEF.

3) Reproducibility:

Protein patterns in IEF change continuously as a function of time and voltage, due to the changes in CA distribution [Fig.2 of (16)]. It follows that pattern reproducibility is difficult to obtain since it depends on critical control of experimental conditions, inclusive of batch identity of CAs. In contrast to band position, the pH of the focused zone in IEF is highly reproducible. However, the methods needed to determine the pH of

the zone in IEF are sufficiently indirect and/or complex to reduce the degree of precision of pH measurement. In all but one method of pH determination on the steady-state zone, one needs to derive pH measurement and stained zone position from parallel gels or tracks which must be assumed to be identical but in practice never are. In measuring pH by the contact electrode on horizontal slabs, this assumption does not have to be made, since the identical track previously analyzed for pH can be subsequently stained; however, this technique depends on prior knowledge of the time required for attainment of the steady-state. Another technique is to measure pH indirectly by "pI markers" in the gel, but this method is highly inaccurate (see section 7).

IPGEF, by contrast, provides zone positions at the steady-state which are constant between experiments and laboratories within the limits of pH gradient reproducibility. If one defines that reproducibility of pH gradient for a particular set of Immobiline reagents and a particular mode of pH gradient formation and polymerization of the IPG gel, a high degree of reproducibility has been claimed (10,17), although not statistically verified. But reproducibility of pH gradients necessarily decreases to the degree that batch variability of Immobilines and their concentrations exists, and to the degree that different laboratories, or the same laboratories at different times, use different gradient making and polymerization techniques. As yet, a data base for quantitating reproducibility between laboratories and over time periods does not exist.

It even appears rather unlikely that reproducibility of zone positions between laboratories will ever come to pass, for the zone positions in IPGEF depend on a) Immobiline purity, concentration and stability on storage either as single reagents or in a mixture; b) accurate dispensing of Immobiline volumes to form the mixtures by which the gradient gel is made; c) the technique of gradient formation and the precision of the internal

dimensions of the cassette; d) the degree of incorporation of Immobilines into the gel matrix. f) If furthermore gels are stored, either intact or in the dehydrated state, their changes on storage introduce another element of irreproducibility.

a) The reproducibility of Immobiline reagents with regard to both purity and concentration is, of course, entirely out of control of the investigator and within the responsibility of the manufacturer, as long as the chemical composition of Immobiline remains a trade secret. Furthermore, it is not known at this time to what degree IPGEF patterns depend on Immobiline storage conditions, such as temperature, the presence of oxygen, pH, as well as on the question whether different Immobilines need to be stored separately or can be stored in mixture. It is also important to know whether storage requires the presence of polymerization inhibitors in some (alkaline ?) or all Immobiline species, and if yes, what the effect of the inhibitors on the polymerization of the IPG gel is. The single available study on Immobiline stability (18) does not answer all of these questions.

c) Among techniques to make linear gradients, the use of 2-chamber gradient mixers (19) is prevalent. The major competitive techniques use computer-directed burets (17) or very simple pumping arrangements (20). One would a priori assume that both a rather sophisticated method of gradient formation like the buret method, or an extremely simple method like the pumping method, are the most reproducible. Of further importance for gradient reproducibility are the precision of the cassette dimensions and the mode of filling (one or several entrance ports, filling from the top vs. from the bottom). At this time, the evidence is still insufficient to decide which of the various modes of cassette filling is optimal. Moreover, the filling of less than 1 mm thick cassettes consisting of one hydrophilic and one hydrophobic surface gives rise to a wavy surface of the rising polymerization mixture during either upward or downward

filling of the cassette.

d) Since polymerization depends on the concentrations of polymerization catalysts and inhibitors, as well as the temperature of polymerization and on the concentration and purity of monomers, a comparison between the reproducibilities of the 3 major gradient making techniques would have to use identical conditions for polymerization inclusive of the control over all of these factors. Further totally unknown factors affecting the composition of the Immobiline copolymer are: i) The question whether all Immobilines incorporate to an equal degree into polyacrylamide; ii) the question as to the total degree of incorporation; iii) the question whether each species is incorporated evenly or asymmetrically along the polymer chain; iv) the question whether the incorporated Immobiline groups are able to interact. The presently recommended polymerization procedure for IPGEF gels has not been developed in response to those questions but is based solely on a study of polymerization kinetics of individual Immobiline species to give homopolymers (21). It is assumed on the basis of that study that each Immobiline species is incorporated 85% by the recommended procedure of polymerization in the presence of dissolved oxygen at 50^{o}C using solely persulfate as a free radical donor. A reproducible Immobiline gel matrix will depend on quantitative conversion of all monomers, including Immobilines, into the polymer. Deaeration of the polymerization mixture and optimization of initiator species and concentrations should help to approximate such quantitative conversion. The low reaction rates of at least some of the Immobilines compared to acrylamide make it even more imperative to convert monomers to polymer efficiently: Even a low percentage of residual unpolymerized acrylamide does not guarantee, in view of these differences in reaction rates, that a particular Immobiline is incorporated with anything near the same efficiency.

It is concluded that reproducible zone positions and patterns

are exclusively obtainable by IPGEF and ICAPG-EF, not by IEF. However, statistical data concerning gradient and pattern reproducibility in IPGEF are not available as yet. Certainly, the advent of commercially prepared Immobiline gels should shift the burden of reproducibility into the realm of industrial quality control and be able to at least provide batches of relatively highly reproducible pH gradient gels.

4) Volume, ionic strength and entry of the sample:

As a first approximation, sample volume limitations appear apparatus dependent and independent of the selection of either IEF or IPGEF as a focusing method. In both methods, applied in either vertical slabs or tubes, there exists no limit to sample volume in the ideal case. In reality, the instability of the pH gradient in IEF does set such a limit, however, for 2 reasons: The sample can migrate into the gel only as long as the changing pH at the gel terminus provides sufficient mobility at the appropriate side of its pI. Secondly, the pH profile must remain steep enough to allow for an approximation of the isoelectric zone by the sample. By contrast, the stable pH gradient in IPGEF is not only compatible with large sample volumes, but even with a continuous introduction of sample into the system. This preparative advantage has not been exploited as yet. The method may be limited in IPGEF by inadequate migration rates out of the sample zone (see below). This problem may be remedied by the accelerated transport in ICAPG-EF, however possibly at the price of swelling problems (section 8).

Horizontal slab apparatus is rather limited in regard to sample volume. Most horizontal apparatus is being used with sample volumes of 2 to 50 µl depending on gel thickness. This limitation is largely independent of the technique in sample application which in IEF is preponderantly by paper strip onto the gel surface, and in IPGEF by sample slots. To avoid the

inconvenience of slot forming during polymerization, a silicone rubber strip with intermitttently spaced holes placed onto the gel surface has also been found useful in IPGEF (17).

In principle, IPGEF suffers from less restrictions in sample ionic strength than IEF (10). The rationale for that advantage is that in IEF the electrophoretic removal of salt leads to a displacement of CAs and consequent shift of the pH gradient. This mechanism obviously cannot apply to IPGEF where charged groups are fixed. In reality, however, the limited transport and the very low current in IPGEF does not allow for an effective desalting of the sample, with the consequence that protein migration from the sample zone is retarded. Furthermore, on horizontal slabs, the discrepancy between the conductance of the sample zone and the surrounding gel is increased at elevated ionic strength of the sample, so that the distortion of the electric field due to that discrepancy increases. Since in gel tubes or in the corresponding isolated tracks of a horizontal slab (10,22) the field is forced to pass through the sample zone, the desalting efficiency is increased when IPGEF is carried out in those modes. The high tolerance for sample ionic strength expected for IPGEF is only realized in ICAPG where conductance is sufficient for an effective removal of the salt from the sample zone, with generation of high enough voltage in the sample zone to allow for sample entry into the gel.

IPGEF appears to present problems of sample entry. These problems have led to a practice of loading several times more protein onto otherwise equivalent IPGEF and IEF gels. In part, these problems probably derive from the zone spreading and the consequent dilution of the protein within the zone which is due to the distortion of the electric field passing through a more conductive protein zone located within a less conductive gel. As already mentioned, this effect can to some degree be reduced by cutting slab gels into separate tracks, or by use of gel tubes. A second relevant factor is the inadequate rate of ion transport

in a medium of exceedingly low conductance. Furthermore, the low ionic strength of the medium leads to protein aggregation and insolubility , an effect that possibly may be remedied by the addition of CAs or detergents to the sample (23).
Non-quantitative sample entry is not usually encountered in IEF, and is overcome by the addition of CAs in ICAPG-EF.

5) Experimental ease:

There can be no question that IEF excels in the ease of experimental procedure over IPGEF, considering that in IEF the pH gradient forms automatically ("naturally") in the electric field once CAs have been added to the gel, and where polymerization efficiency is of little consequence. This discrepancy in experimental ease is particularly acute for the presently conventional mode of IPGEF involving the following steps: a) Preparation of the light and heavy Immobiline mixtures; b) pH neutralization of the Immobiline mixtures, c) gradient formation by gradient mixer, d) polymerization at 50°C, e) washing, f) drying to the original weight and g) pre-electrophoresis. A more recent simplified procedure omits steps b, e, f, g), and replaces c) and d) by simpler procedures, replacing the gradient maker by a pump and allowing for polymerization at room temperature or in the cold (20). Although simplified, that procedure however does not attain the degree of procedural simplicity achieved by IEF. This difference may, however, cease to exist once commercially available prefabricated IPGEF gels will be introduced.

6) Ease of pH gradient design:

One of the key advantages of IPGEF over IEF is the ease with which the pH gradient can be computed on the basis of the known pKs of the functional groups of Immobilines and of their volume ratios (7,17). By contrast, since the chemical identities of the

CAs in IEF are unknown, pH gradients can only be modified by trial-and-error to find a specific desired pH gradient. Even when the CAs are replaced by simple buffers, which allows one to compute steady-state pH gradients, realistic predictions seem hard to obtain: Those programs which neglect diffusion but are capable of rapidly and inexpensively estimating pH gradients with up to 20 constituents provide insufficiently accurate predictions (24) while those which consider diffusion require computer simulation techniques and correspondingly more computing capacity and time (25) which makes it economically prohibitive to predict pH gradients with a practically useful number of 20 - 50 buffer constituents.

However, the predicted properties of pH gradients in IPGEF also fail to depict experimental ones with sufficient accuracy: At least in the wide (pH 4 to 10) pH range, the predicted even conductance across the pH gradient cannot be verified by experiment (12). The discrepancy of experimental from predicted pH gradients is less visible in view of the logarithmic nature of pH. No data demonstrating statistically reproducible IPGEF pH gradients are available as yet (as discussed above), and thus no rigorous testing of the accuracy of the predictions is possible. Nonetheless, one can state <u>a priori</u> that the computation of pH gradients neglects charge interactions between functional groups which must exist in reality. The argument that only 1 in 50 residues carries a functional group, and that therefore charge interactions are impossible, is fallacious since it neglects the stereochemistry and the folding of polymer chains. Secondly, inaccuracy must result from failure to incorporate 100% of monomers into the polymer, in conjunction with the fact that the % incorporation must vary among monomers with different functional groups.

Notwithstanding all of these reservations, it remains clear that simple predictability of pH gradients by computer simulation is one of the real advantages of IPGEF over IEF.

7) Susceptibility to pH and voltage measurement:

Upon the introduction of IPGEF, it was taken for granted that in view of the low ionic strength of the gel, pH could not be directly determined by contact electrode. Instead, users of the method have applied indirect ways to determine the pH gradient, based on the steady-state positions of protein pI-markers. However, the accuracy of such indirect methods of pH measurement is difficult to ascertain since it depends on knowing the pI of the markers at the temperature and ionic strength and the viscosity of a gel, on their attainment of the steady-state as well as on the reagent purity and history of the protein markers. Furthermore, the assumption of a curve shape between adjacent markers and a curve defined by a small number of markers makes this technique rather unreliable. Our laboratory succeeded in taking direct pH measurements on IPG gels, using a relatively wide (5 mm diameter) contact electrode, and by attaching a water droplet to the electrode and a very long measurement time, up to 30 min at some pHs (20). This, of course, is not only impracticably laborious but also inaccurate. The problem of direct pH measurement was only solved in ICAPG gels where the addition of CAs adds sufficient conductance to the gel to allow for a rapid measurement by contact electrode, comparable to that in IEF.

It is concluded that direct pH gradient detrmination is one of the key advantages of both IEF and ICAPG-EF over IPGEF.

In the practice of focusing, field strength measurement is far less important than pH measurement. It becomes important in trouble shooting, in addition to its theoretical interest. With regard to field strength measurement, IEF, ICAPG-EF and IPGEF present qualitatively the same problem of requiring a high ("infinite") impedance voltmeter. In all 3 cases, reliable voltage measurement implies ascertaining that the field strength

values, added across the pH gradient gel, add up to give the value of the applied potential across the gel; and that the voltage profile across the gel be the same whether or not the measurement proceeds progressively from either the anode or the cathode (20).

8) Perturbation by gel hydration and dehydration phenomena:

Water extrusion from the gel during electrofocusing is not a problem in tube or vertical slab apparatus, but presents a problem for horizontal slab gels in ICAPG-EF and, to a lesser degree, in IPGEF. If the water exudates occur in the gel region of interest, they will tend to produce diffuse zones. If they are not, they appear harmless, and the effect can be neglected as long as the gel surface is wiped intermittently during electrofocusing.

A second hydration-dehydration phenomenon of importance is the formation of "ridges" on the gel surfaces of horizontal IEF and ICAPG-EF gels. Presumably these "ridges" present osmotically swollen zones of CAs. Although the "ridges" are visible in horizontal slabs only, the must be equally present in vertical slab or tube gels where they presumably give rise to the observed irregular gel swelling along the gel length after gel removal from the glass walls. In vertical apparatus, this differential gel swelling along the pH gradient leads to a weakening of adherence of the gel to the glass walls, with corresponding need to strengthen wall adherence by coating of the glass with polyacrylamide or agarose, by mechanically supporting the gel (e.g. by Nylon mesh) and by its hydrostatic equilibration through maintenance of equal liquid levels of anolyte and catholyte (26). The "ridging" is not observed in IPGEF, except at concentrated protein zones when the protein content is sufficient to cause the osmotic adsorption of water into the zone.

The extent to which "ridging" may affect protein patterns is

unknown; it may have a significant effect on the local field strength. The relative prevalence of hydration problems appears to be highest in ICAPG-EF, less in IEF and least in IPGEF.

9) Artefacts:

Very little evidence exists as to the degree of hydrophobic, ionic or H-bond interactions between proteins and the charged residues (CAs and Immobiline respectively) of either an IEF or an IPG matrix. For IEF, all 3 types of interactions have been demonstrated (27). Evidence also exists for ionic and H-bond interactions in IPG gels (10). With regard to the former, IPG should be more vulnerable than IEF in view of its lower ionic strength. One would speculate, however, that hydrophobic interactions should be less on IPG gels at least compared with IEF gels in the alkaline range (28).

10) Compatibility with SDS-PAGE:

The coupling of EF with SDS-PAGE to give 2-D gels is one of the important applications of EF. In that application, IEF gives rise to interactions between basic and hydrophobic CAs, neutral detergent (29) and SDS. The interaction products traverse and perturb the 2nd dimensional SDS-gel by introducing into it a field asymmetry, Joule heating problems and different voltage gradients and migration rates. These perturbations must be expected to be absent in IPGEF, assuming that the IPG gel does not contain any non-covalently adsorbed Immobiline or other mobile ionized species. Nonetheless the transfer of protein zones from IPGEF into the SDS-gel has been found to be inferior to that from IEF (30). This may be due to a) electroendosmosic interference with the implantation of the IPGEF-strip into the SDS-matrix (22), and/or b) to a greater insolubility of the zones which have approached the pI more closely, or have electrofocused

at the steady-state for a longer time, than in IEF (31). a) The negative charging of the IPGEF-strip in a basic SDS-buffer seems responsible for producing a contact problem between strip and SDS-matrix [Fig.1 of (22)]. Transfer may also be impeded by the cathodic direction of the electroendosmotic flow. The contact problem appears largely remedied through an increase in the viscosity of the IPGEF gel by the addition of glycerol to the equilibration medium prior to strip application to the SDS-gel (22). b) This interpretation is also consistent with the beneficial effect of 30% glycerol in the IPG gel (22) since protein insolubilization may be reduced in that medium. Such interpretation may also be related to the higher potential applied in IPGEF compared to IEF, causing a greater extent of isoelectric precipitation.

One of the most important applications of the 2-D procedure concerns the resolution of water-insoluble membrane proteins. In that application, the EF gel has to contain dissociating agents and detergents capable of maintaining the separated proteins in solution; the most frequently used agents are 9 M urea, 2% NP-40 (alias Triton X-100) (32). For reasons of micellar size and inhomogeneity, it appears preferable to exchange CHAPS or β-octylglucoside for the NP-40 (33). The question then arises, whether or not IEF and IPGEF are equally compatible with mixtures such as 9 M urea, 0.01-0.05 M CHAPS. This should be the case in view of the insensitivity of zwitterionic detergents to ionic strength in the range of 0 to 0.2 M [Fig.7 of (34)].

On balance, IPGEF and IEF appear to be both burdened with problems upon coupling with a 2nd SDS-dimension, although the nature of those problems is different. Weighing one set of problems against the other, it appears impossible at this time to judge which one is more perturbing to the 2nd dimensional gel pattern.

11) Ease of protein isolation, preparative load capacity and

purity of product:

Preparative IEF can be conducted in a variety of gel media such as Sephadex (dextran), agarose, in addition to polyacrylamide, as well as in density gradients and in free solution, using various types of apparatus. This versatility in preparative approaches does not exist for IPGEF which is restricted to a polyacrylamide matrix. However, IPGEF has the preparative advantage of avoiding the presence of CAs in the fractionated zone and thus eliminates the problems of separating them from the protein. Preparative load capacity is mainly determined by 4 variables: a) The cross-sectional area of the gel; b) the degree of distribution overlap between the zone of interest and its nearest neighbors; c) the water content of the gel (35); d) the limits imposed by the buffering capacity of the CAs and/or Immobilines respectively. In these regards, the sole preparative advantage of IPGEF is that sample can be introduced to the limit posed by item c), without consideration of focusing time and even in continuous fashion, as pointed out in section 4. Furthermore, in horizontal IPGEF gels the swelling of isoelectric protein zones creates a "water pocket" with enhanced capacity (36). To accelerate preparative focusing, ICAPG-EF appears to be the preferred method, unless an interaction between protein and CA perturbs the resolution or product purity.

Subsequent to separation by either IEF or IPGEF, zones of interest need to be excised, and protein needs to be freed of the gel by either diffusion or electrophoresis. In both cases, extraction of the protein by electrophoresis appears more efficient. Numerous procedures and apparati for extraction in a continuous or discontinuous buffer system are available; the latter has advantages with regard to product concentration (e.g. 37,38). A popular procedure embeds the slice to be extracted into a horizontal gel and transfers the protein electrophoretically into a trough containing a suitable adsorbent

(hydroxyapatite or ion exchange medium) (35). A very simple isolation procedure which avoids zone excision in IPGEF has recently been described, whereby the desired component is collected within a trough cut in the gel at the position of its pI; the trough is filled with suspension of Sephadex in water (39).

Product impurity problems in IEF and IPGEF seem comparable with regard to the polyacrylamide-derived non-proteinaceous impurities which must be removed by gel filtration at 0°C in a subsequent step (40,41). Even IEF conducted in an agarose gel gives off enough carbohydrate impurity to require such a final purification step (ibid.). In view of this requirement, any contamination of the product with CAs in either IEF or ICAPG-EF becomes less important. But a quantitative removal of charged small contaminants like the CAs or amino acids by gel filtation is not possible - such removal requires an electrophoretic step.

It is concluded that in view of water accumulation in the focused zone, and its tolerance for continuous or repetitive sample application and consequent enrichment of the focused zone, IPGEF has a greater load capacity than IEF. Quantitative data concerning that advantage have been reported (36).

12) Equipment needs and cost:

Contrary to wide prejudice, the cost of Immobilines per EF separation is equivalent to that of CAs. However, IPGEF is undoubtedly the more expensive method in view of the need for a gradient former and a high-voltage power supply capable of delivering at least 100 V/cm of gel, and preferably as much as 500 V/cm. Since either 2-chamber gradient mixers, or proportioning pumps (20) are commonly available in biochemical laboratories, the need for a gradient forming device can be discounted. For constructing specific non-linear pH gradients, specialized apparatus is required (9). With the development of

improved Joule heat dissipation techniques, such as the introduction of horizontal slab apparatus cooled by Peltier cells (e.g. Isobox, Hoefer Instruments), high field strength will become a routine way for increasing resolution in other forms of gel electrophoresis as well, so that the need for a 5,000 to 10,000 V power supply in IPGEF will become increasingly non-specific.

The only other equipment - pH contact electrode, infinite impedance voltmeter and micro-ammeter - is also equally needed for IEF and IPGEF.

It is concluded that equipment needs for IPGEF are greater than those for IEF. This difference may disappear with time.

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Footnote

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Stains for the Detection of Proteins Separated by Electrophoretic Methods

The development of electrophoretic techniques has been paralleled by development of Protein detection methods with ever-increasing sensitivities. Detection techniques have progressed from direct observation of protein-coated microspheres and colored proteins, requiring milligram amounts, to the detection of proteins and by their absorption of ultraviolet light, the observation of schlieren patterns, labelling with radionuclides, by direct staining with organic, fluorescent dyes, and most recently by silver stains which require a tenth of a nanogram. Many detection techniques may be employed quantitatively, provided that their methodological limitations are respected. Most protein stains and autoradiographic methods exhibit protein-specific quantitative optical density/concentration relationships, which are indicative of the dependence of detection methods on the content of specific groups within each protein. These protein-specific staining relationships may be utilized to differentiate proteins, and emphasize the need to limit quantitative comparisons to homologous proteins. So long as intergel studies are confined to comparisons of homologous proteins and observations are made within the nonsaturation range of the detection procedures, valid quantitative results may be obtained.

1. Historical Introduction

The observations in 1807, that colloidal particles migrate in electrical fields, by the physicist Ferdinand Frederic Reuss, initiated the development electrophoretic separation techniques [1]. Applications of electrophoretic methods to problems of protein and nucleic acid purification would not have been possible without complementary detection methods. The earlist applications of electrophoresis relied on direct observations of objects including, cells, colloidal particles, bacteria, and the naturally colored proteins such as, myoglobin, hemoglobin, ferritin, and cytochrome c [2]. However, most protein molecules could not be observed directly with visible light and their electrophoretic properties were studied by observing quartz microspheres with the proteins

adsorbed to their surface [3-5].

Detection of non-colored proteins by the specific absorption of ultraviolet light was first demonstrated by Tiselius in the 1930s [6]. Despite its early introduction, protein detection by ultraviolet absorption has never gained widespread acceptance, primarily because it requires a special light source, filters, and optical components that are transparent to ultraviolet light. Tiselius also utilized schlieren, or shadows, created by boundaries between regions with different refractive indices due to the varing concentrations of proteins in electrophoretic systems as a detection method [6]. This system also required complex optical systems.

Introduction of organic stains for detection of proteins and eliminated many of the complications inherent in the earlier systems and often provided increased sensitivities. The use of moist filter paper as an electrophoretic support medium or carrier for zonal electrophoretic separation stimulated the adaptation of a number of histochemical stains for the detection of uncolored proteins [7]. These stains were usually employed after the proteins were "fixed" or made immobile, to decrease protein loss in the staining solutions. Heat, 110°C after electrophoresis was one of the first methods used to "fix" proteins. Early organic protein stains included Bromophenol Blue [8] and Amido Black [9]. Lipoproteins were preferentially stained by Oil Red O [10], while glycoproteins were detected by a red color that was produced upon their oxidation with periodic acid and subsequent reaction with fuchsin sulfurous acid (Schiff's reagent) [11]. Coomassie Blue stains, with their capability of detecting as little as half a microgram of protein, are the most sensitive of these organic protein stain [12]. This increased sensitivity of the Commassie Blue stains was originally used to detect proteins separated on cellulose acetate. It was also complimented the increased protein resolution of acrylamide gel electrophoretic methods. Most of the early organic stains were employed to detect proteins after their electrophoretic separation, post-electrophoretic stains. Fluorescent protein stains which were introduced by Talbot and Yaphantis in 1971 [13] can now detect as little as one nanogram of protein [14]. However, these fluorescent stains usually require reaction conditions that are best performed prior to

electrophoresis, pre-electrophoretic stains, and their formation of covalent bonds with the protein molecules generally alters the charge of the proteins [15]. This charge alteration is not of consequence for electrophoretic techniques that separate proteins on the basis of molecular weight, such as sodium dodecyl sulfate (SDS) electrophoresis, but it can alter separations by isoelectrofocusing [15].

Radioactively labelled proteins may be visuallized without staining by autoradiographic methods which were first introduced by Becquerel and Curie in their discovery of the phenomenon of radioactivity [16], or by fluorographic techniques which were introduced by Wilson to study tritium labelled metabolites involved in photosynthesis [17]. Proteins radioactively labled to a high specific activity can be detected with sensitivities equal to, and often better, than those obtained by the most stains. However, the use of radioactively labled proteins is limited, as it is difficult to achieve high specific activities in animal studies and unethical in reseach involving humans.

Introduction of silver as a general protein stain increased the sensitivity of protein detection to a tenth of a nanogram [18-19]. This was a 100 fold gain over that attained by the most sensitive commonly used organic stain, Coomassie Blue. The first silver stains used for detection of proteins separated by polyacrylamide gel electrophoresis were adapted from histological silver stains. These were often tedious, requiring three hours of manipulations and use of numerous solutions [18-19]. In the seven years since the introduction of silver staining as a general method for the detection of proteins in polyacrylamide gel electrophoresis numerous staining proptocols have been developed [20]. These protocols can be divided into three categories: the diamine or ammoniacal silver stains, the non-diamine stains including stains based on photographic chemistry, and stains based on the photo-development or photo-reduction of silver ions to form the metallic silver image. Some of the newer silver stains can be performed in less than fifteen minutes and they may be employed for quantitative analyses.

2 Post-Electrophoretic Organic Protein Stains

2.1 Commassie Blue stains

Coomassie Blue stains are the most commonly used organic stains for post-electrophoretic detection of proteins separated on polyacrylamide gels. They were originally developed as acid wool dyes and they were named "Coomassie dyes" to commemorate the 1896 British occupation of the Ashanti capital, Kumasi or "Coomassie", now in Ghana. In a collaborative effort to find a highly sensitive protein stain that could be used in quantitative studies, a microbiologist of the Australian National University, Fazekas de St. Groth, and textile chemists at the University of New South Wales School of Textile Technology (Australia) tested numerous dyes and dyeing techniques [21]. Their studies demonstrated the intense protein staining abilities of the triphenylmethane Coomassie stains.

Coomassie Brilliant Blue R250 (the letter "R" stands for a reddish hue while the number "250" is a dye strength indicator) was the first of the triphenylmethane stains to be introduced. It can detect as little as 0.5ug/cm of protein and gives a linear response up to 20ug/cm. However, it should be noted that the relationship between stain density and protein concentrations varied for each of four proteins tested [21]. Fazekas de St. Groth et al. originally introduced this stain to detect proteins on cellulose acetate, agar or starch gels [12]. Meyer and Lamberts (1965) adapted the Coomassie Brilliant Blue R250 stain for polyacrylamide gels [21]. They used electrophoresis to remove excess stain, but a number of proteins displayed some mobility during this destaining [21-23]. Non-electrophoretic methods of destaining have largly eliminated this problem. Other Coomassie stains, such as Coomassie Brilliant Blue G250 ("G" indicates that this stain has a greenish hue), have augmented the stain introduced by Fazekas de St. Groth et al.. Coomassie Brilliant Blue G250 has a diminished solubility in 12% TCA. permiting its use as a colloidal dispersion. Such a colloidally dispersed dye does not penetrate gels, permitting rapid staining of proteins without an undesired background [24]. Another derived Coomassie stain, Coomassie Violet R150, has gained some favor by virtue of its ability to rapidly stain proteins on polyacrylamide gels and its ease of destaining [25-26]. Although Coomassie Violet R150 is

no longer produced by Imperial Chemical Industries, Ltd., a very similar dye, Serva Violet 49, is available. Serva Violet 49 differs from Coomassie Violet 150 by the substitution of a diethylamine group for a dimethylamine group. As Imperial Chemical Industries, Ltd. no longer produces Coomassie Blue R250 or G250. They still hold the trademark "Coomassie", so that manufacturers who are currently producing Coomassie type dyes have had to introduced their own trademarked names [27].

2.2 Mechanism of Coomassie Brilliant Blue Staining

Coomassie Blue staining requires an acidic medium for electrostatic attraction to form between the dye molecules and the amino groups of the proteins. This ionic attraction, together with van der Waals' forces, binds the dye-protein complex together. The binding, however, is fully reversible by dilution under appropriate conditions [12]. The relatively high staining intensity of Coomassie Blue stains, compared to other organic dyes, is apparently due to secondary bonds formed between dye molecules. Additional dye may be bound by dye-dye interactions to dye molecules that are ionically bound to, or in hydrophobic association with, protein molecules [28].

Recent studies concerning the mechanisms of Coomassie dye staining of proteins have indicated the importance for the basic amino acids. Righetti and Chillemi noted that polypeptides rich in lysine and arginine were aggregated by Coomassie G dye molecules, suggesting that the dye interacts with the basic groups in the polypeptides [29]. Studies of proteins with known sequences, by Tal et al., have confirmed these observations and demonstrationed of a significant correlation between the intensity of Coomassie blue staining and the number of lysine, histidine and arginine residues in the protein [30].

2.3 Metachromatic effects

Secondary dye-binding or dye-dye interactions may play a fundamental role in the metachromatic effects that have been observed with some polypeptides that are stained with Coomassie Brilliant Blue R250 [31-32]. Some

peripheral nerve proteins stain to produce both red and blue bands with Coomassie Brilliant Blue R250 after separation by SDS-polyacrylamide electrophoresis. Calf skin collagen and histone-1 protein display similar red-staining bands, as do most other collagen samples [32]. Metachromatic shifts are often observed when dye molecules are stacked together [33]. Such dye stacking or close aggregation may affect the dye's electron resonance structure, resulting in an altered response to excitation by light.

These metachromatic shifts are affected by a number of variables. Bands containing more than 5 ug of a protein that normally produces a red hue have been shown initially to stain blue in the center of the band. Only with continued destaining does the red become apparent. Red-staining proteins such as histone-1 or collagen may be converted to blue bands by placing the gels in solutions containing alcohol, SDS or TCA [32]. Metachromasy is also diminished by staining at elevated temperatures [31]. The dependence of metachromic effects on protein specificity, concentration, temperature, solvents, and the type of spectral shift observed with Coomassie Blue are characteristic of metachromatic mechanism rather than staining artifacts due to dye contaminants.

2.4 Other Common Post-Electrophoretic Organic Protein Stains

Amido Black (Acid Black 1) and Fast Green (Food Green 3) are commonly utilized for protein detection following polyacrylamide gel electrophoresis. However, Coomassie Blue R250 staining is three times the intensity of Fast Green and six times the intensity of Amido Black staining [27]. The staining intensities of these dyes are approximately proportional to their relative molar adsorption coefficients. Wilson [28] estimated that 1 mg of protein will bind approximately 0.17 mg of Amido Black, 0.23 mg of Fast Green, 1.2 mg of Coomassie Blue R250 and 1.4 mg of Coomassie Blue G250. It is unlikely that this variation is due to variations in molecular weight between these dyes since their weights only range between 616.5 daltons for Amido Black and 854.0 daltons for Coomassie Blue G250. Variations in binding must be due to an differences in the number of dye molecules bound per protein molecule. The higher staining intensity

Coomassie Blue G250 may be due to this dyes higher efficiency at forming dye-dye interactions or hydrophobic interactions between the dye molecules and the proteins. Secondary binding mechanisms also occur with Amido Black and Fast Green dyes, although perhaps not at levels observed with Coomassie Blue R250, as these dyes display metachromic effects with certain proteins similar to the metachromasy observed with Coomassie Blue R250. Amido Black produces blue-green bands with certain histones rather than its characteristic blue-black color, while Fast Green produces a difference in the ratio of blue to green hues [34].

3 Pre-Electrophoretic Organic Protein stains

Pre-electrophoretic organic stains usually involves the covalent binding of either a fluorescent or colored residue to the protein prior to electrophoresis. Potential advantages of these stains include: the possibility of performing stoichiometric reactions with proteins without the diffusion limitations imposed by staining within a gel matrix, the process of electrophoresis can be followed visually with "stained" proteins, and there are no background problems due to dye-trapping or reaction of the dye with the gel. An often cited disadvantage of covalently bound pre-electrophoretic stains is that they usually alter the charge of proteins [15]. This objection is not of consequence for sodium dodecyl sulfate (SDS) electrophoresis, as the protein's mobility depends on molecular weight, and the dye molecules are usually too small to produce an appreciable effect. Furthermore, as long as the stains react with proteins in a stoichiometric manner, shifts in protein pattern should be highly reproducible, permitting construction of valid protein maps and protein identifications.

3.1 Non-fluorescent Stains

Remazol Brilliant Blue R, was the first anionic dye used for prestaining proteins [35]. A major disadvantage was its lower limit of sensitivity (3ug of protein). Bosshard and Datyner introduced anionic dyes with sensitivities comparable to the Coomassie Blue stains, Drimarene Brilliant

Blue K-BL and Uniblue A [15]. These dyes are capable of detecting bands containing as little as 0.5 ug of protein. They react primarily with the amino groups of proteins as well as the hydroxyl groups of serine and tyrosine. A similar range and sensitivity has been achieved with a cationic dye that binds to the protein's amino groups, causing less alteration in isolectric focussing patterns than anionic dyes [36].

3.2 Fluorescent stains

Fluorescent stains are currently the most sensitive pre-electrophoretic stains in use. The first fluorescent stain used to visualize proteins in gels was anilinonaphthalene sulfonate, a post-electrophoretic stain which is thought to bind to a protein's hydrophobic sites, to form a fluorescent complex [37]. Its limit of sensitivity is about 20 ug of protein. Pre-electrophoretic fluorescent staining with dansyl chloride was first introduced by Talbot and Yaphantis [13]. This stain reacts with proteins to form fluorescent derivatives in 1-2 minutes at 100°C, with a sensitivity limit of 8-10 ng.

Reagents that were first designed to increase the detection limits of amino acid analyzers have resulted in a number of highly sensitive flourescent stains [38]. The first of these, fluorescamine, is a non-fluorescent compound however at room temperature and alkaline pH, it reacts with primary amines of amino acids within to yield a fluorescent derivative. It has proven capable of detecting as little as 6 ng of myoglobin [39-40]. A related compound, 2-methoxy-2,4-diphenyl-3(2H)-furanone (MDPF) has the same speed and simpicity of reaction as fluorescamine, and its protein derivative is 2.5 times as fluorescent as a fluorescamine-labelled protein. Furthermore it does not fade as rapidly. As little as 1 ng of protein has been detected with MDPF. It also has a linear response from 1-500 ng. As with most other protein stains, a plot of relative fluorescence versus protein concentration revealed a different slope for each of four proteins studied [14].

Although these fluorescent stains achieve greater sensitivity than other organic stains, they require ultraviolet light for visualization, and

direct quantitation requires fairly sophisticated equipment. These problems, coupled with the altered electrophoretic mobility during isolectric focussing (a result of the altered protein charge(s) from the fluorescent derivatization), has tended to inhibit utilization of these fluorescent stains.

4 Silver Stains

Silver nitrate, the main ingredient in silver stains, was first described by the Arabian alchemist, Gabir Dschabir ibn Hajjam in the eighth century. The observation that this compound has the ability to blacken when in contact with organic substances, including human skin, is usually credited to Count Albert von Bollstadt [41]. Modern applications of this property of silver nitrate began with Krause's 1844 observation that silver nitrate solutions could be used to stain small pieces of fresh tissue [42]. Golgi and Cajal used silver stains to establish the foundations of modern neuroanatomy [43]. Cajal also adapted photochemical methods to develop new histological silver stains that produced better definition of nerve fibers. In general, since the time of Cajal, histological silver stains have become more complex as empirical alterations have been made in the procedures to increase the specificity of the staining for specific cells or subcellular structures.

Introduction of silver as a general stain for proteins separated by polyacrylamide gel electrophoresis permitted the detection of as little as 0.1 ng of protein. This silver stain, an adaptation of a histological silver stain, was demonstrated to be 100-fold more sensitive than previous stains [18-19]. Other histological silver stains adapted for protein detection were limited to the detection of specific proteins, modifying subgroups, or to proteins electrophoresed in agarose. A modified Gomori silver stain was used to study phosphorylase [45], while another histological silver stain, originally developed to visualize nucleoli, was used to detect nucleolar proteins. Kerenyi and Gallyas adapted a histological silver stain to visualize cerebrospinal fluid proteins separated on agarose [47-48]. This stain did not achieve widespread acceptance, perhaps because it produced numerous staining artifacts, did

not work well in polyacrylamide, or because of a report that it was quantitatively irreproducible [49]. However, recent work by has improved this stain's performance in agarose [50].

In the seven years since the introduction of silver staining as a general method for the detection of proteins sparated by polyaccrylamide gel electrophoresis numerous staining protocols have been developed. These protocols can be divided into three basic catogories: the diamine or ammoniacal silver stains, the non-diamine chemical development silver stains, and the silver stains that depend on light for the photoreduction of the silver ions to form the metallic image.

4.1 Diamine Silver Stains

These stains rely on the stabilization of the silver ions by the formation of silver diamine complexes, with ammonium hydroxide. Diamine silver stains were first developed for the visualization nerve fibers [51]. Silver ion concentrations are usually very low in these stains, as most of the silver is bound in diamine complexes [52]. Diamine stains tend to become selectively sensitive for glycoproteins if their concentration of silver ions is decreased. This specificity can be minimized by maintaining a sufficient sodium to ammonium ion ratio in the diamine solution [53]. However, in some applications, an emphasis of the diamine stains specificity has proven useful, as in the adaptation of a diamine histological silver stain to visualize neurofilament polypeptides in electrophoretic analyses of spinal cord homogenates [54].

In the diamine stains, the ammoniacal silver solution must be acidified, usually with citric acid, for image production to occur. The addition of citric acid lowers in the concentration of free ammonium ions, thereby liberating silver ions to a level where their reduction by formaldehyde to metallic silver is possible. The optimal concentration of citric acid also results in a controlled rate of silver ion reduction, preventing a non-selective deposition of silver.

4.2 Non-Diamine Chemical Development Silver Stains

Most of the non-diamine chemical development silver stains were developed by adapting photographic photochemical protocols [55-59]. These stains rely on the reaction of silver nitrate with protein sites in acidic conditions, followed by the selective reduction of ionic silver by formaldehyde in alkaline conditions. Sodium carbonate and/or hydroxide and other bases are used to maintain an alkaline pH during development. Formic acid, produced by the oxidation of formaldehyde, is buffered by the sodium carbonate [60].

4.3 Photo-development Silver Stains

Photo-development stains utilize energy from photons of light to reduce ionic to metallic silver. Scheele in 1777 recognized that the blackening of silver chloride crystals, by light was due to the formation of metallic silver [61]. This ability of light to reduce ionic to metallic silver was adapted by William Fox Talbot, in 1839, as the basis of a photographic processes that dominated photography from its introduction until 1862, when photo-development was replaced by "chemical development" processes [62]. The use of photo-reduction provides for a rapid, simple, sensitive silver stain method for detecting proteins [63-64].

Most chemical development stains require a minimum of two solutions, in addition to the fixing solution. This requirement for multiple solutions is a result of the use of alkaline solutions for the chemical reduction of silver. The presence of silver ions and an organic reducing agent in an alkaline solution often results in the uncontrolled reduction of silver. However, since light can reduce silver in an acidic solution, a photo-development stain may utilize a fixation solution followed by a single staining solution. Such single-solution photo-development silver stains have two major advantages over chemical-development silver stains. First, pH gradient effects are eliminated. In chemical development, one solution, containing silver ions, diffuses out of the gel, while the solution containing the reducing agent diffuses into the gel. The interactions of these solutions creates complex pH gradients within the gel. A single-solution photo-development stain reduces such diffusion effects, minimizing staining artifacts due to variations in gel thickness.

Proteins on ultra-thin supporting membranes such as cellulose nitrate stain poorly with the "chemical stains" because they retain very little silver nitrate when transferred into alkaline solution for image development. Because the photo-development stain contains the silver ions in the image-developing solution, proteins may be visualized even when bound to thin membranes.

4.4 Combinations of Photo-Development And Chemical-Development Stain

By combining photo-development and chemical-development methods, a stain has been developed which can detect proteins and nucleic acids in the nanogram range, it can be performed in under fifteen minutes, and it results in minimal background staining [65]. This stain utilizes: a silver halide, to provide a light senstive detection medium, and to prevent the loss of silver ions from membranes or thin layer plates; photo-reduction, to initiate the formation of silver nucleation centers; and chemical-development, to provide a high degree of sensitivity by depositing additional silver on the silver nucleation centers (formed by the photo-reduction of the silver halide). This stain displays an average detection sensitivity of 1 ng of protein or 10ng of DNA. The stain's rapidity of action, and its' ablility to stain samples spotted on membranes, such as cellulose nitrate, has afforded the opportunities to investigate aspects of the mechanism of silver staining.

The first step in this stain protocol employs copper acetate, a metal salt that is both a good fixative [66] and a silver stain enhancer. The mechanism of copper's stain enhancement, in this and other silver stains, may be similar to its action in the biuret reaction [19], in which a characteristic color shift, from violet to pink, is achieved by titrating peptides in the presence of copper ions. Copper complexes formed with the N-peptide atoms of the peptide bonds are primarily responsible for this reaction. There are also some number of secondary sites which may interact with copper. Any elemental copper formed may displace positive silver ions from solution as copper has a greater tendency to donate electrons than silver, indicated by its position in the electromotive series of the elements. Following the treatment with copper acetate, the membrane is

sequentially soaked in a solution containing chloride and citrate ions and then in a solution containing silver nitrate. The membrane is then irradiated with light while it is in the silver nitrate solution. The presence of the resulting silver chloride, in the membrane, produces a significant increase in light sensitivity over that which can be achieved with silver nitrate alone. Herman Vogel, a 19th century photochemist, suggested that although silver chloride is more sensitive to the reducing action of light than silver nitrate, it is fixed in position by its insolubility and the potential density of its image would be limited unless the free silver ions supplied by the silver nitrate are present to diffuse into the photo-reduction centers [67]. This increase in sensitivity was further enhanced in this stain by the presence of acetate and citrate ions [64,67-68]. White fluorescent light proved to be the most effective for this photo-reduction. Ultra violet light produced a denser image, but it also produced an unacceptable background stain. Continued irradiation with white light would provide sufficient photo-reduction to produce an image of the protein pattern on the membrane, however, photo-reduction alone usually results in a dense background stain when applied to thin membranes [64]. By limiting the light irradiation to a total of four minutes, only enough to initiate the formation of a latent image, formation of a visiable image is achieved by chemical-reduction. The chemical-reduction of ionic to metallic silver was effected by placing the membrane in a solution containing the reducing reagents hydroquinone and formaldehyde. During image formation, ionic silver is reduced to metallic silver, formaldehyde is converted to formic acid [60] and hydroquinone to quinone. Unreacted silver chloride is removed from the membrane, to prevent a grayish cast background, and continued darkening of the membrane as the silver ions in the unreacted silver chloride are photo-reduced, by exposure to light. Removal of the silver chloride is accomplished by complexing the silver chloride with sodium thiosulfate to form a series of complex argentothiosulfate sodium salts, most of which are soluble in water [62]. The argentothiosulfate sodium salts, unreacted reagents, and silver grains formed in solution that may have precipitated onto the surface of the membrane are washed away with water.

4.5 General Silver Stain Mechanisms

The basic mechanism underlying all protein detection silver stains involves reduction of ionic to metallic silver. Detection of proteins in the gel or membrane requires a difference in the oxidation-reduction potential between the sites occupied by proteins and adjacent sites of the gel or membrane. If a protein site has a higher reducing potential than the surrounding gel or matrix, then the protein will be positively stained. Conversely, if the protein site has a lower reducing potential than the surrounding gel or matrix, the protein will appear to be negatively stained. These relative oxidation-reduction potentials can be altered by the chemistry of the staining procedure. Proteins separated on polyacrylamide gels have been shown to stain negatively if the gel is soaked in the dark in silver nitrate followed by image development in an alkaline reducing solution (such as Kodak D76 photographic developer). By treating the gel with potassium dichromate prior to the silver nitrate incubation followed by development of the image in an alkaline reducing solution (utilizing formaldehyde as the reducing agent), a positive image is produced [58]. Positive images may also by obtained by substituting potassium ferricyanide [54], potassium permanganate [69], or dithiothreitol [59] for the potassium dichromate in this stain. Dichromate, permanganate and ferricyanide are thought to enhance the formation of a positive image by converting the protein's hydroxyl and sulfhydryl groups to aldehydes and thiosulfates, thereby altering the oxidation-reduction potential of the protein. Although the formation or presence of aldehydes has often been suggested as essential for silver staining, in certain histological stains neither aldehyde-creating or aldehyde-blocking reagents appreciably affect silver staining [70]. Dithiothreitol, a reducing agent, also creates a positive image, perhaps by maintaining the proteins in a reduced state. However, other reducing agents, such as beta-mercaptoethanol, do not enhance positive image formation. Alternatively, all of the positive image enhancing compounds may form complexes with the proteins and these complexes may act as nucleation centers for silver reduction [20].

4.6 Reactive Groups on Proteins which affect Silver Stains

The combination photo-development, chemical-development silver stain has been utilized to study amino acid homopolymers and individual amino acids to gain information about reactive groups that may be involved in silver staining reactions [65]. The only individual amino acids which stained were cysteine and cystine. Poly-methionine and the hydrophilic basic amino acid polymers: poly-lysine, poly-arginine, poly-histidine, and poly-ornithine also stained [65]. Staining of the basic amino acids in their homopolymeric form, but not as individual amino acids, may be related to the shift of pKs that is normally associated with the incorporation of amino acids into peptides. This shift in pK toward the neutral range results in an increased presence of ionized amino acid side chains closer to the physiological pH. The ability of reactive group in an amino acid side chains to form complexes with metal ions may be enhanced by such a shift. For example, a shift in the pk of an amino group would reduce the proton competetion that a metal ion must overcome for the amino group's N-atom electron pair. Staining of the basic amino acid and methionine homopolymers, but not their individual amino acids may also indicate the need for cooperative effects of several intramolecular functional groups to form complexes with the silver or copper ions [71].

Heukeshoven and Dernick also observed silver staining of the basic homopolymers of histidine, arginine, and ornithine, although they did not report staining of the basic amino acid homopolymer poly-lysine [72]. Furthermore Nielsen and Brown noted that the basic amino acids: lysine, arginine, and histidine, (in both a free and homopolymeric form) produced colored complexes with silver [73].

Heukeshoven and Dernick reported silver staining of the homopolymers of glycine, serine, proline and aspartic acid [72] while Nielsen and Brown reported the formation of colored silver complexes with: aspartate, and tyrosine [73]. Staining of these homopolymers was not observed in the study of Merril and Pratt [65]. In this regard prior metal binding studies have failed to demonstrate metal interactions with the side-chain hydroxyl groups of serine, threonine or tyrosine [71]. These discrepancies

concerning the non-basic amino acids may be due to differences in the staining procedures employed; the Heukeshoven and Dernick study stained homopolymers on polyacrylamide gel, while Nielsen and Brown studied formation of silver-amino acid complexes in solution. Both of these studies used formaldehydye in an alkaline sodium carbonate solution for image development, while Merril and Pratt utilized acidic conditions and a combination of light, hydroquinone and formaldehyde for image formation [65].

The importance of the basic and the sulfur containing amino acids has been corroborated by observations with purified peptides and proteins of known amino acid sequence [65]. Leucine enkephalin, which has neither sulfur containing nor basic amino acids does not stain with silver, while neurotensin which also has no sulfur containing amino acids but does have three basic amino acid residues (one lysine and two arginines) does stain. Gastrin produced a weak staining reaction. It lacks basic amino acids but it has one sulfur containing amino acid, methionine. Oxytocin stains fairly vigorously. It also has no basic amino acids but it does have two sulfur containing cysteines. The staining reaction of angiotensin II was rather anomalous. It produced a negative stain rather than a positive silver stain despite its two basic amino acids, arginine and histidine. All the other polypeptides; insulin somatostatin, alpha-melanocyte stimulating hormone, thyrocalcitonin, aprotinin, vasoactive intestinal peptide and ACTH, contained both basic and sulfur containing amino acids and they all produce positive silver staining reactions [65].

The importance of the basic amino acids has been further substantiated by evaluations of the relationship between the amino acid mole percentages of proteins and their ability to stain with silver. The best correlations were achieved when comparisons were made between the slopes of denatured proteins staining curves and the proteins mole percentages of basic amino acids, particularly with histidine and lysine [65]. A similar correlation has been observed by Dion and Pomenti [74]. Dion and Pomenti suggested that this correlation may be due to an interaction between lysine and glutaraldehyde, which was used in their stain protocol. The bound glutaraldehyde could supply aldehyde groups to facilitate the reduction of

ionic silver. While this mechanism may play a role in the stain protocol employed by Dion and Pomenti, it is are unlikely to be a factor in the Merril and Pratt protocol [65], since that protocol did not employ glutaraldehyde. Dion and Pomenti also suggested that alkaline conditions may be important for the formation of silver complexes with lysine and histidine. However the Merril and Pratt protocol utilized acidic conditions. No significant correlations have been found between protein amino acid mole percentages and their ability to stain with silver in a native, or undenatured state [65]. This lack of a significant correlation with undenatured proteins is probably due to the inaccessibility of many of the potentially active amino acid side chains in the undenatured protein structures.

The observation of a significant correlation between silver staining intensity and the mole percent of lysine is most likely due to the reactive "amino group" at the terminus of lysine's side chain. The amino group has been known to have significant metal binding ability due to its strong electron-donor qualities and the ligand-field effect of its nitrogen atoms [71]. However, amino groups involved in peptide bonding and N-terminal amino groups are in themselves insufficient for visualization with silver stain. If they were capable of independently reducing silver ions, all peptides, proteins, and amino acids would stain positively. Amino groups involved in peptide bonding and N-terminal atoms may of some importance for the intensity of the stain, as these atoms have been observed to form 13 different complexes with copper between pH 1.5 to pH 11.0 [75]. Copper may be reduced under the conditions of some stain protocols and then be displaced by silver. Alternatively, silver may also interact directly, but weakly, with these groups.

The contribution of histidine to silver staining is not surprising, since the imidazole groups in the histidine side-chains are often important for metal-binding in the metalloproteins. The effectiveness of histidine in metal binding is probably due to the fact that imidazole groups are good electron donors [71]. The enthalpy changes in the formation of metal-nitrogen (imidazole) bonds are only slightly less than those found with metal-nitrogen (amino) bonds [76]. The slightly lowered ability of the

imidazole group, relative to the amino group, to donate electrons for the formation of metal complexes may be balanced by imidazole's lower pK. The lower imidazole group's pK, in contrast to the higher pK of an amino group, reduces the metal ion's competition with protons for the imidazole's nitrogen atom's electron-pair [71].

The guanidine group in arginine's side chain has proven to be less active than either the amino or the imidazole groups in the side chains of lysine and histidine respectively. Arginine's correlation coefficient was not found to be significant in studies of comparing staining densities to mole percent of arginine. This lack of activity of the guanidine group may have been, in part, responsible for the negative staining reaction of the peptide angiotensin II which contains the two basic amino acids arginine and histidine (one residue of each). However, neurotensin, which contains two arginine residues and one lysine residue, stained well. Cooperative metal binding effects between active groups may play a role in the staining process. In angiotensin II the arginine residue is separated from the histidine by three residues, while in neurotensin the two arginines are adjacent to each other and only one residue separates them from a lysine residue.

Of the nonpolar and uncharged polar amino acids, only the sulfur containing amino acids, methionine, cysteine and cystine, showed any silver staining reactivity with the Merril; Pratt Protocol [65]. Cysteine and cystine were the only amino acids to stain as an individual amino acids and they may account for the silver staining properties of the peptide oxytocin. Oxytocin contains no basic amino acids and its only sulfur containing amino acids are two cysteine residues. The ability of cysteinyl side-chains to form complexes with silver ions is well known. At the low pHs utilized in this protocol, the predominant species is $Ag(HCys)_2^+$,[71]. It has been suggested that the ability of reducing agents [including: thiosulfates, sulfides, borohydrides, cyanoborohydrides, mercaptoethanol, thioglycolic acid, cysteine, tributylphosphine reducing metal salts (such as $FeCl_2$,$SnCl_2$ and $TiCl_3$) and dithiothritol] to intensify silver stains may be related to the generation of thiol groups in cysteine residues [77]. However, proteins that contain no cysteine or proteins with an alkylated

cysteine(s) were also affected by these reducing agents in some stain protocols [72].

Methionine's ability to participate in the silver staining process has been demonstrated by silver staining of methionine homopolymers. Methionine may also be responsible for the staining of the peptide gastrin. Gastrin contains no basic amino acids and only one methionine residue. In general the thioether sulfur atoms in the methionine residues are weaker electron donors than the sulfhydryl sulfur atoms in the cystiene residues. The only metal ions that have been observed to bind to the thioether's sulfur atoms are those with electrons in the d^8 and d^{10} configurations (Pd^{++}, Pt^{++}, Ag^{+}, Cu^{+}, and Hg^{++}). The affinity of sulfur ligands for metal ions may be explained by the higly polarized state sulfur atoms achieve during interactions with small metal ions containing high charge densities. Sulfur's electron distributions and energies enhance the enthalpies of metal ion bonding (they have high crystal field stabilization energies). There may also be electron resonance bonding in the metal-sulfur bond [71]. Insignificant staining correlations were observed between staining densities and mole percentages of the sulfur containing amino acids methionine and cysteine [65]. This observation may indicate a relatively minor silver staining role in proteins containing large numbers of basic amino acids. However, this poor staining correlation is somewhat of a paradox since poly-methionine stained with a higher silver density than the basic amino acid homopolymers [65]. This paradox may be explained by a strong requirment for cooperative effects between sulfur atoms and silver atoms which may be disrupted in heteropolymers.

5 Properties Of Silver Stains

5.1 Color Effects

Most proteins stain with monochromatic brown or black colors. However, the ability of silver to produce colors was first noted when certain lipoproteins tended to stain blue while some glycoproteins appeared yellow, brown or red in a study of cerebrospinal fluid proteins [78]. This color effect is most likely an analogue of a photographic phenomena first

described by Herschel in 1840 [58,79]. Herschel noted in 1840, that if he projected the spectrum of visible light obtained by passing sunlight through a prism onto a silver chloride-impregnated paper, the colors of the spectrum appeared on the paper, particularly a "full and fiery red" at the focal point of the red light [79]. Since these observations by Herschel it has been found that the color produced depends on three variables: the size of the silver particles, the refractive index of the photographic emusion or electrophoretic gel, and the distribution of the silver particles. In general, studies with photographic emulsions have shown that smaller grains (less than 0.2 microns in diameter) transmit reddish or yellow-red light, while grains above 0.3 microns give bluish colors, and larger grains produce black images [68]. Modifications of the silver staining procedures, such as lowering the concentration of reducing agent in the image development solution, prolonging the development time, adding alkali, or elevating the temperature during staining often enhance color formation. Some silver stain protocols have been developed to produce colors that may aid in identification of certain proteins [73,80-81]. Production of color with silver stains depends on many variables. Amino acid side groups play a major role in color formation [73]. Furthermore, variations in protein concentration and conditions of image development may also produce color shifts. Color-enhanced silver stains tend to become saturated at low protein levels and often produce negatively stained bands or spots. These factors tend to make quantitative analysis with silver stain protocols that enhance color effects more difficult.

5.2 Protein Specific Silver Stains

Silver stains can demonstrate considerable specificity. Hubbell et al. stained nucleolar proteins with a histological stain [46], while Gambetti et al. adapted a silver stain specific for neurofilament polypeptides [54]. Many silver stain protocols detect not only proteins but also DNA, [82-84], lipopolysaccharides [85], and polysaccharides [86]. In a study of erythrocyte membrane proteins, sialoglycoproteins and lipids stained yellow with a silver stain protocol, while other membrane proteins counterstained with Coomassie Blue [87]. All silver-stains do not detect proteins such as calmodulin or troponin C. However, pretreatment with gluteraldehyde often

permits positive silver staining of these proteins [88]. Histones may also fail to stain with silver. Fixation with formaldehyde coupled with simultaneous prestaining with Coomassie Blue partly alleviates this problem. However, even with this fixation procedure sensitive for histones is decreased 10-fold compared with detection of neutral proteins [89]. Another example of differential sensitivity was demonstrated in a study utilizing four different silver stain protocols to stain salivary proteins. Different protein bands were visualized with each of the stains [90].

5.3 Quenching Of Autoradiography by Silver Stains

Quenching of ^{14}C-labelled proteins is minimal with most of non-diamine silver stains and even the most intense diamine stained, ^{14}C-labelled proteins can be detected by autoradiography with only a 50% decrease in image density. This loss of autoradiographic sensitivity can generally be compensated for by longer film exposures. However, detection of ^{3}H-labelled proteins is severely quenched by all silver stains. Destaining of the silver stained gel with photographic reducing agents can often permit detection of as much as half of the fluorographic density of ^{3}H-labelled proteins, providing that the initial staining was performed with a non-diamine silver stain. Many diamine stains continue to quench, even after treatment with photographic reducing agents, so that fluorographic detection of ^{3}H-labelled proteins is not feasable. This impediment to 3H detection with diamine stains is likely to be due to a greater amount of residual silver deposited in the gels by the diamine stains, which block the weak-beta emissions from ^{3}H. Residual silver has been demonstrated in gels that have been cleared by photographic reducing agents by the faint silver image of the protein can be observed after drying the gel with heat. Silver has also been demonstrated in these "cleared" gels by electron beam analysis [91].

5.4 Sensitivity

Silver stains offer the most sensitive non-radioactive method for detecting proteins separated by gel electrophoresis. They are 100-fold more sensitive than the Coomassie stains for most proteins [18-19]. Chemical-development

silver stains are in general, more sensitive than photo-development silver stains. This loss in senstivity may be compensated for by the ability of photo-development stain to produce an image within 10 to 15 minutes after gel electrophoresis [64]. Attaining high sensitivities with silver stains requires care in selecting reagents, small traces of contaminants may cause a loss of sensitivity and result in staining artifacts. Artifactual bands with molecular weights ranging from 50 to 68 kiloDaltons have commonly been observed in silver stained gels. Evidence has been presented indicating that these contaminating bands are due to keratin skin proteins [92]. Water used to make solutions should have a conductivity of less than 1 mho.

6 Quantitation With Protein Stains

6.1 Quantitation Using Organic Stains

Fazekas de St. Groth et al. demonstrated an accuracy of plus or minus 10% in measuring the concentration of a protein in the range of 0.5 - 20 ug/cm using Coomassie Blue R250 [12]. He noted that, although individual proteins displayed linear relationships between absorbance and concentration, the slopes differ for each protein. This variation in Coomassie Blue staining now appears to be related to the mole percent of the basic amino acids in the protein [30]. Therefore, a standard curve must be produced for each protein assayed and quantitative comparisons limited to equivalent protein spots on two-dimensional or equivalent protein bands on one-dimensional electrophoretograms. This is especially true for metachromatically staining proteins, which often display an additional complication, in that their bands fade more rapidly than the non-metachromatically staining proteins. In one study with Amido Black, certain metachromatic histone bands faded by 30-50%, while other protein bands in the same gel faded by only 5-15% [34]. This fading reflected a disproportionate loss of stain, rather than a loss of histone protein, since the bands could be restained. In the case of smaller proteins such as insulin, decreases in band densities have been demonstrated to be due to the loss of protein during staining [12]. Another problem in utilizing Post-electrophoretic organic stains occurs because diffusion of the dye into the gel is slow. This results in "ring-dyeing" if staining is terminated prematurely [15]. In "ring-dyeing", the stain

concentration is less in the center of a band or spot than at the edge, due to the insufficient diffusion of dye molecules. Regional nonstoichiometric processes, as occur in "ring-dyeing", require careful control of staining parameters to assure that they are not present in protein bands or spots that are to be quantitatively analyzed.

6.2 Quantitation Using Silver Stains

A reproducible relationship between silver stain density and protein concentration has been found with most silver stain protocols. The linear portion of this relationship extended over a 40-fold range in concentration, beginning at 0.02 nanograms per mm^2 for most proteins [19,57-58,63]. Protein concentrations greater than 2 ng/mm^2 generally cause saturation of silver images, resulting in non-linearity above that concentration. Saturation can usually be recognized by bands or spot with centers which are less intensely stained than the regions near the edges. This effect is similar to the "ring-dyeing" noted with some of the organic stains. An often quoted report by Poehling and Neuhoff [93] states that "Silver does not stoichiometrically stain proteins, unlike Coomassie Blue". However, their own silver-stain data actually is linear over a 30-fold range in protein concentration, while their Coomassie Blue data is only linearity over a 20-fold range [20,57].

Employment of Curve-fitting techniques, as described by Coakley and James [94] for the analysis of curvilinear relationships found in the Folin-Lowry method of protein estimation [95] may be utilized for quantitative analysis of silver stain curves. With careful measurement of total stain densities, estimates of relative protein concentrations have been made over a 220 fold concentration range with six purified proteins [65].

Plots of silver stain densities versus protein concentrations produce different staining curves for each proteins studied [18,57-58,65]. Protein specific staining curves have also been observed with the organic stains, including Coomassie Blue [30,12],as noted above, and with most protein assays such as the commonly used Lowry protein assay [95]. The observation that each protein produces a unique density verses concentration curve in

these studies, illustates a dependence on specific reactive groups contained in each protein. Furthermore, the occurance of protein-specific curves argues against a stain mechanism that depends on some fundamental subunit common to all proteins, for example the peptide bond, or a unique element in each protein, such as the terminal amino acid. A stain that depended on a subunit, such as the peptide bond, would result in similar staining curves for all proteins, when the density of staining for each of the protein bands or spots was plotted against the mass of protein contained in each of the bands or spots. Similarily, a stain that was based on a reaction with a unique element in each protein, for example the terminal amino group, would produce similar plots for each protein when the stains densities were plotted against the number of molecules contained in each band or spot. It is possible that these protein-specific curves may be utilized to differentiate proteins and to provide insights concerning the reactive groups responsible for the staining reactions. The importance of the basic amino acids, particularly lysine and histidine as discussed in the section "Staining of Peptides and Proteins with Known Sequences" illustrates the use of these proteins specific staining curves. It indicates the need for a careful choice of a "standard protein(s)" if this stain is used quantitatively to estimate protein consentrations. A protein containing an abnormal large number of stain reactive groups would produce a curve which would tend to underestimate the concentration of proteins containing normal numbers of reactive groups. A similar correlation between the intensity of Commassie Blue staining and the number of basic amino acids in proteins [29,30] caused Tal et al. to suggest the use of egg white lysozyme rather than the more commonly used bovine serum albumin as a protein standard. This suggestion is based on their observation that the basic amino acid content of proteins ranges between 10-17 mole percent, with a modal content of 13 mole percent [30]. Egg white lysozyme has a basic amino acid mole percent of 13.2 while bovine serum albumin has a basic amino acid content of 16.5 mole percent. For similar reasons, egg white lysozyme may also prove to be an optimal standard for quantitative silver stain applications.

6.3 Quantitative Inter-gel Protein Comparisons

The occurance of Protein specific staining curves with most staining protocols requires that quantitative inter-gel comparative studies limit comparisons to homologous protein bands or spots on each gel. For example, the actin spot on one gel can be compared with an actin spot on another gel, but not with a transferrin spot. Quantitative inter-gel comparisons requires the presence of reference proteins for the normalization of spot or band staining densities. One scheme for normalization utilizes "operationally constitutive proteins", a subset of proteins contained in each gel that have constant intra-gel density ratios to each other in all of the gels in a study. The sum of the densities of the "operationally constitutive proteins" in an arbitrarily designated "standard gel" are compared with the sums of the densities of the constitutive proteins in all other gels, and a specific normalization factor is determined for each gel. These gel specific normalization factors are then utilized to correct the densities of all the proteins on each of the gels to those of the standard gel. This scheme corrects for variations in staining, in image digitization, and initial protein loading; a variation of initial protein loading of up to 10-fold may be tolerated [57,63].

7 Conclusions

The introduction and development of electrophoretic techniques has produced ever more powerful means of resolving proteins from complex mixtures. Development of these separation techniques has been paralleled by the developmentof protein detection methods with ever-increasing sensitivities. Techniques have progressed from direct observation of protein-coated microspheres and colored proteins, to the detection of proteins by their absorption of ultraviolet light, the observation of schlieren patterns, and, more recently, by direct staining with organic, fluorescent and silver stains.

During this technical evolution, sensitivity has increased from the milligram to the tenth of a nanogram level. This range of sensitivity coupled with high resolution separation techniques, now permits clinical studies of proteins in body fluids that were not possible even a decade ago. Similar sensitivities can be achieved with proteins labeled to high

specific activities with radioactive tracers, however, raadioactive labelling is often not possible in animal and human studies for economic and, in the latter case, ethical considerations.

Many of the protein staining techniques may be employed quantitatively, provided that their methodological limitations are respected. Most protein stains and autoradiographic methods exhibit protein-specific quantitative responses. Optical density/concentration relationships are usually linear over a thirty- to forty-fold range in concentration and quantitation may be extended beyond this range by the use of curve fitting techniques. Protein-specific staining slopes are indicative of a dependence of these staining methods on the content of specific groups within each protein. These protein-specific staining slopes may be utilized to differentiate proteins, and emphasize the need to limit quantitative comparisons to homologous proteins. So long as intergel studies are confined to comparisons of homologous proteins and observations are made within the linear range of the detection procedures, valid quantitative results may be obtained.

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Peptide Separations: Techniques Reviewed

Numerous techniques, both electrophoretic and chromatographic have been developed to separate peptides from complex mixtures. However, detection of low concentrations of small molecular weight peptides in biological material, as well as separation of proteolytic digests of larger proteins continues to offer difficulties. Various chromatographic techniques such as paper chromatography, paper electrophoresis, thin layer chromatography, thin layer electrophoresis, polyacrylamide gel electrophoresis and high performance liquid chromatography have been employed for the analysis of peptides. But each of these techniques has limitations in terms of the range of peptide sizes that can be successfully separate. Many of the methods also lack the ability to adequately separate structurally related molecular species, such as intermediates in the biosynthetic pathway and posttranslationally modified peptides[1].

1 Paper Chromatography and Electrophoresis

Since the 1944 introduction of paper chromatography by Consden, Gordon, and Martin, it has proven to be a simple chromatographic technique. It readily provides qualitative information and depending on the method of detection it is possible to obtain quantitative data.

Samples to be analyzed are usually dissolved in organic solvents with low boiling points such as acetone, ethanol, or chloroform [3]. Ionic and polar molecules separate fairly well in this system, however its ability to separate nonpolar molecules is limited [4]. Solvent systems containing butyl alcohol and acetic acid or systems containing amyl alcohol are used for separation of basic peptides, whereas pyridine is a good solvent for the separation of acid peptides [4]. For highly efficient separation of peptides on paper it is important for the analyzed substances to be free of larger proteins, free amino acids and inorganic ions [4]. These interfering substances may cause poor

resolution, spot distortions, and aberrations in reactions used for peptide detections [5]. Humidity in the chromatographic chamber and often the complexity of the peptide mixture may also affect the reproducibilty of the Rf values for peptides in individual solvent systems [4].

Many types of papers are used for peptide analysis: standard cellulose, papers containing carboxyl groups, ion-exchange resins, cellulose ion-exchange, alumina or silica gel adsorbents and glass fiber papers [3]. There are four methods of applying solvents based on the direction of solvent movement; ascending, descending, horizontal, and radial techniques [3]. These techniques may use any one of three different types of solvent systems for their development: an aqueous stationary phase; a stationary hydrophilic organic solvent ; or a stationary hydrophobic organic solvent [3].

Ultraviolet light is primariy used for the detection of peptides on paper [3]. In addition, peptides may be detected by: enzymatic detection [3]; ninhydrin staining [5,6]; production of a fluorescent product with fluorescamine [7] or o-phthaldehyde [5]; reaction with the Ehrlich reagent which detects tryptophan and tyrosine residues; and radioactivity using a labelled modifier [8]. Dintrophenyl [DNP] amino acids can not be sucessfully separated by paper chromatography since they have a tendency for trailing, and also their migration on paper depends on the quantity of amino acids applied and on the presence of other DNP amino acids [9].

High voltage paper electrophoresis is a rapid and reproducible method for the separation of peptides. Not only is this procedure faster than paper chromatography, the samples need not be desalted unless very large samples are used, or unless the salt concentration is much higher than physiological [10]. However, this procedure requires special attention to such details as pH, temperature, voltage,and type and length of paper. This method uses the same types of papers as those described for paper chromatography, also the detection methods of

peptides are similar. As little as 1-2 nmoles of peptides may be detected by fluorescamine staining after high voltage electrophoresis on paper [11].

Although, this procedure is highly useful for the resolution of small peptides, it does have several drawbacks such as: heat dissipation and evaporation of buffer from the paper electrophoretagram [10]; the inability to examine peptides that have low solubility in the electrophoretic bufer; and the trailing of intermediate and larger size peptides on the paper [11].

Many researchers used a two dimensional system of paper chromatography followed by high voltage paper electrophoresis to analyze tryptic digests of various proteins [12-15]. This system is very reproducible. Katz [14] found that ascending chromatography with n-butanol/acetic acid/water [4:1:5] followed by electrophoresis with a pyridine acetate buffer at pH 3.7, allowed one to detect the replacement of a single amino acid. This chromatography system separated the polar from the nonpolar peptides, and the electrophoresis separated the polar peptides according to charge.

A number of researchers feel that the most reproducible method of peptide separation involves electrophoresis on paper followed by paper chromatography [16,17]. Ingram [17] using paper electrophoresis at pH 6.5, followed by ascending chromatography in n-butanol/acetic acid/water [3:1:1] found this technique also allowed for the detection of a single amino acid difference between normal and sickle cell hemoglobins of man.

2 Thin Layer Chromatography and electrophoresis

Although the principle of thin layer chromatography [TLC] was described by Izmailor and Schraiber in 1938 [17], it was not until 1956 that it was introduced as a technique for analytical adsorption chromatography [18].Thin layer chromatography is a relatively inexpensive, fast, and simple method which can separate complex mixtures on a single thin

layer plate. This method can be used for the determination of approximate molecular weights of peptides and small molecular weight proteins. In addition, this procedure can be used to determine the purity of peptides as well as for the analysis of peptides when only minute amounts are available [19]. Also the starting material, the intermediates, and the end products in peptide synthesis can be examined rapidly by TLC [20].

Once TLC plates have been prepared , the technique of chromatography is similar to that used for paper chromatography. Commercially prepared plates are available, they offer the advantage of highly uniform layers and good reproducibilty. The following types of prepared plates are available: silica gel Kieselguhr, alumina [acid, basic, or neutral], magnesium silicate, calcium sulphate, zirconium phosphate, and hydroxylapaptite [21]. Organic materials used on the plates include: various derived cellulose such as carboxymethyl, diethylaminoethyl, phosphate and various impregnated cellulose such as polyethylenimine impregnated; Sephadex, free and substituted; Avirin and Avicel which are microcrystalline celluloses; polyamides such as nylon and perlon; and powdered ion-exchange resins [21].

Thin layer chromatography depends on differences in charge, molecular weight, hydrophobicity, and adsorption behaviors for the separation of peptides [22]. The presence of foreign substances as well as sample overloading can cause poor resolution, spot distortions, and aberration in colored reactions used for peptide detections [5].

Like paper chromatography, TLC permits rapid qualitative results, but exact quantitative determinations are difficult to obtain. Peptides can be located on TLC plates by UV spectroscopy [5], ninhydrin staining [23], fluorometry [23], radioautography [5] and dinitrophenylation [24]. Location of these peptides with ninhydrin, fluorescamine or o-phthaldialdehde permitted the detection of picomole quantities of peptides [23]. It is important to note that only peptides detected using fluorescamine can be recovered from their layers without destruction of any amino acids thereby allowing the peptide to be

subjected to further analysis [23,24,25]. Detection of peptides with ninhydrin is limited because the molar responses decrease with increasing chain length, and because N-acylated peptids are usually nonreactive. Also it fails with cyclic peptides unless they contain free amino groups in their side chains [8].

Peptides can be isolated from thin layer plates either by solvent extraction and filtration or by electrophoretic elution of the peptides [26]. After elution [22] the fractions can be quantitatively analyzed by ultraviolet spectrometry [27], by fluorimetry [28], by calorimetry after chemical reaction [29] or by weighing [30].

Thin-layer chromatography has been used for the studies of small peptides [30] as well as for peptide mapping [32,33,34,35]. Burzynski [32] found that tritiated dansly chloride labelled peptides analyzed on polyamide plates followed by autoradiography and densitometry allowed detection of 2.5 x 10-14 moles of peptides.

Reverse phase TLC plates made with silanized silica gel impregnated with detergents are also available for the analysis of peptides [33]. The type and concentration of the detergent determines the separations that can be achieved. The interaction of the hydrophobic side chain and functional groups of the detergent with the amino acids controls the migration of the peptide on the plate. Peptides with hydrophilic or basic amino acids run with the solvent front while peptides with one or more hydrophobic amino acids are retained [36]. This reverse phase TLC system [using Whatman KC18F plates] provided a rapid check of HPLC separation, and detection of the material with ninhydrin verified that the optical density peaks were actually peptides [37]. Before polar samples can be analyzed in this reverse-phase TLC system, an ion pairing reagent must be added to the mobile phase. This reduces the migration of the polar solutes and minimizes spreading of the spots [37]. Peptide containing hydrophobic and basic residues have lower Rf values because of their increased interaction with the stationary phase. Therefore, an increase in the amount of acetonitrile or methanol in the mobile phase results in a higher Rf value. As observed

for reverse phase high perforamnce liquid chromatography, acetonitrile was found to give more efficient separation of peptides in reversed TLC than was achieved with methanol [37]. It is possible to use TLC data to develop a more effecient HPLC system, however TLC could still be the desired method when a very large number of samples are to be analyzed.

Two dimensional thin layer methods in which peptides are separated by electrophoresis horizontally followed by chromatography [ascending] is commonly called thin-layer electrophoresis [39]. This procedure has been used widely for peptide map analysis [38,39,40,41]. Thin layer electrophoresis requires only a simple apparatus which provides a cheap and satisfactory alternative to more expensive and sophisticated high performance liquid chromatography machinery. Also, this method does not require the use of high voltage equipment which are capable of dissipating large amounts of heat [42].

3 Polyacrylamide Gel electrophoresis

In 1967, Shapiro and coworkers [43] demonstrated that the migration of denatured polypeptides on polyacrylamide gels correlated closely with their length. However, application of this method for determination of the molecular weight of peptides less than 10,000 on sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE] has encountered many problems. The importance of the intrinsic charge of the peptide on the influence of SDS binding was observed by Swank and Munkries [44]. Assuming that all peptides bind 1.4 mg SDS per mg, the smaller peptides bind relatively less SDS on a molar ratio; therefore the intrinsic charge becomes more important at these lower molecular weights [44]. Also, factors such as shape, final net charge and side chain modifications [44,45] also greatly influence the migratory behavior of peptides on polyacrylamide gels. Many approaches have been used to obtain better resolution of low molecular polypeptides in SDS-PAGE , they include: addition of urea to the gels [44,45,46,47]; increasing the concentration of acrylamide and/or crosslinkers [44,47,48]; altering the buffer composition [44,47]; altering the pH [11,49]; using linear acrylamide gradients [47,50]; and addition of

sucrose [47].

Swank and Munkries were among the first to investigate conditions for the maximal separation of peptides [44]. First, they added urea to the standard SDS system [43]; urea increases the solubility of some peptides, and it also reduces the effective pore size in acrylamide gels. In addition, urea may decrease the size of the detergent micelles. This may help to overcome the problem of coelectrophoresis of small peptides, since peptides less than a certain length all end up in complexes of peptides and detergent of the same size and charge [51]. And finally the presence of urea allows one to use higher levels of cross-linker which improves resolution of oligopeptides [44].

The Swank and Munkrie system makes use of higher acrylamide concentrations which improved the separation of peptides [44]. Another advantage came from the replacement of sodium with Tris, a cation with much lower mobility thus less heat is produced in the system. Tris may also contribute a small amount of buffering capacity to the system at pH 6.9 [44].

The major drawback of this system is the long running time which results in diffuse protein bands, thus decreasing resolution. The broadening of protein bands also results from the use of a uniform separation gel without a stacking gel and a continuous buffer system. And finally this system does not allow for accurate moleclular weight determinations between 10,000 and 20,000.

Although Swank and Munkries [44] developed a procedure for separating peptides between 1000 and 10,000 daltons on tube gels, this method was not applicable to slab gels [45]. This led Burr and Burr [45] to modify this technique with the addition of stacking gel to allow separation of peptides between 1500 and 10,000 daltons using slab-cast gels. Linearity beteen mobility on the gel and molecular weight of the proteins was observed to 25,000 daltons.

More recently, a system which depended on buffer composition and

concentration and which was not critically dependent on acrylamide concentrations gave accurate molecular weight determinations for low molecular weight proteins from approximately 2500 to at least 90,000 [52]. The separating gel contained 5 to 18% acrylamide, all with 5% crosslinker concentrations. Reproducible results required 8M urea in the separation gel, omitting urea or lowering the concentration of urea gave inaccurate molecular weight determinations for proteins between 10,000 and 20,000. However, the improved resolution of this system is primarily due to the buffer system used. The buffer of the stacking gel contains a relatively low-mobility acetate ion, and the buffer of the separating gel contains the high mobility strong anions, sulfate and chloride, as the leading and trailing ions respectfully. The proteins will remained stacked as long as their mobilities are between those of the leading and trailing ions. The organization of the ions, acetate, sulfate, and chloride, at the beginning of electrohoresis determines the unstacking of the lower molecular weight proteins. The sulfate/acetate/protein complex is stacked but as electrophoresis procedes the chloride ion overtakes the complex, the acetate and proteins then become successively unstacked [52]. This discontinuous buffer system also allows one to load dilute samples on the gel since stacking results in concentration of the sample. Furthermore, electrophoresis is complete within 4-5 hours thus limiting the amount of diffusion of the polypeptides within the gel.

Kyte and Rodriguez [53] also developed a method for separating peptides that contains a stacking gel; this system controls the stacking of the proteins by altering the pH. In this system the acrylamide concentration was not found to be critical, the length of a peptide was approximately the same when determined using 12.5 and 20% polyacrylamide gels. However the Ferguson plot analysis indicated that the gel with the higher percent acrylamide gave the more accurate peptide length. These investigators determined that higher concentrations of protein added to the gels caused the occurence of electrophoretic artifacts and diffuse protein bands [53]. The major drawback of this system is that it only provides high resolutin for peptides between 25 and 250 residues in length and a large majority of

the peptides to be studied contain less than 25 residues.

More recently a SDS-PAGE system using a 10 to 18% linear gradient containing 7M urea with an acrylamide:bisacrylamide ration of 20:1, a 0 to 10% sucrose gradient, and Laemlli discontinous buffer [54] was described [47]. Proteins ranging in molecular weights from 1500 to 25,000 were highly resolved. Although the electrophoresis running time is long [15 h] the high crosslinking ratio lowers the diffusion of the peptides in the gel as well as limits the leaking of the polypeptide from the gels during staining [47].

Two polyacrylamide gel electrphoretic systems which do not contain detergent were described for the separation of peptides.[49]. The acidic system contains 50% acrylamide, has a pH of 3.0 and containes urea in the stacking gel but not in the resolving gel. Peptides with one positive charge to about 180 daltons were resolved. To separate peptides which have a net negative charge at pH 3.0 or are insoluble at this acidic pH, an alkaline peptide gel was used [49]. This gel has a pH of 9 and also has urea in the separating gel. This system has been used to study histone proteins [55].

As early as 1973 iodinated and uniodinated Angiotensins were separated on gel electrophoresis on the basis of charge [56]. Recently a polyacrylamide gel electrophoresis system which separated Angiotensin I, II, and III based entirely on net charge rather than size was demonstrated [50]. The system was run a pH 6.41, positive polarity, used gel concentrations ranging from 24 to 40% acrylamide, 2% bisacrylamide and used a discontinuous buffer system.

Two dimensional electrohoresis has also been used to separate peptides [57]. One must take precautions to prevent protien loss from gels during equilibration and loading steps. If the isoelectric focusing strips are equilibrated longer than one minute prior to loading on SDS slabs there was a significant diffusion and loss of the small molecular weight proteins from the gel strips. The use of a higher percent acrylamide in the first dimension effectively decreased diffusion of

the peptides [57]. Hashimoto et.al. [47] showed that a higher crosslinking ratio [20:1, acrylamide:bisacrylamide] also lowered the leakage of the small peptides from the gels. Blattler and coworkers [48] investigated the acrylamide and bisacrylamide concentration effects on the properties of the gels. Altering bisacrylamide concentration affects maximum pore size of the gel while changing acrylamide concentrations affects the average pore size [48]. They noted that too much bisacrylamide produces weak, paste-like gels that are rigid as well as brittle.Preliminary evidence from Blattler's lab also indicates that catalyst conditions are important for producing uniform quality gels [48].

The small molecular weight peptides which occur in very low concentrations in body fluids that are separated by SDS-PAGE can be analyzed by silver stain [58]. However, rapid diffusion and loss during the staining and destaining procedures may limit quantitative studies of the low molecular weight peptides [11]. But, these peptides may be retained in the gels by fixing them chemically as opposed to methanol-acid denaturation [59]. For example, glutaraldehyde which reacts with primary amino groups and guanidium groups can be used to fix peptides in the gels [57]. It should be noted that peptides cannot be fixed by glutaraldehyde on isoelectric focusing gels and subsequently silver stained because carrier ampholytes are fixed in the presence of glutaraldehyde and will also stain [60]. Formaldehyde [61] can also be used to fix peptides in the gels, but proteins which do not have a free amino group are lost from the gels. [49].

Although the silver staining procedure is much faster than staining with Coomassie Blue, Coomassie Brilliant Blue R-250 is often used to stain peptides between 1500 and 10,000 [45]. Addition of copper sulfate [0,5%] aided in the retention of the peptides in the gel [45]. In addition, since peptides are lost from the gel during acid precipitation, fixation of the peptide with 1.25% glutaraldehyde permitted their use of Coomassie blue procedure to detect peptides with molecular weights as low as 1000 [50].

Other methods of detecting peptides include: autoradiography preceeded by glutaraldehyde fixation [49]; spectrophotometric detection of the tyrosine or tryptophan residues [62]; separation of peptides by isoelectric focusing on polyacrylamide gels followed by fixation on glutaraldehye impregnated paper and identified using immunoperoxidase staining procedures [63]; and fluorescent staining [64]. However, there were problems using fluorescamine. Modifications of primary amines of peptides with fluorescamine increases the net negative charge of the peptide resulting in excessive rapid migration ,therefore higher acrylamide concentrations [12-16%] were necessary to separate peptides from the fluorescent front [64]. Also pH affects the mobility and resolution of peptides modified with fluorescamine [11], only at the intermediate pH [8.9 to 9.4] are the peptides well resolved without trailing. At lower pH the peptides move too slowly to achieve significant separation whereas at higher pH values they move too fast, producing trailing.

4 High Performance Liquid Chromatography

High performance liquid chromatography [HPLC] has become a very important analytical tool for isolation and separation of peptides for biological, chemical, and biophysical studies. Reliable HPLC systems were developed based on peptide charactereristics such as length of the peptide chain, hydrophobicity, and nature of the individual amino acids [65]. The column supports have a definite pore structure which depends on the type of liquid chromatography [ion exchange, size exclusion, or partition/adsorption] being considered [66].

High Performance Liquid Chromatography offers distinct advantages over classical chromatography such as increased resolution, speed, reproducibility, and high recoveries of limited quantities of samples. High sensitivity may also be achieved when the peptides are detected by radioimmunassay, fluorometrically or spectrophotometrically. Since amides absorb in the 210-230 nm region of the spectrum, polypeptides may be detected spectrophotometrically thereby achieving high sensitivity (50 to 100 nanograms). Another advantage of HPLC is that

it is nondestructive thereby allowing recovery of biological materials or recovery of material desired for sequencing.

4.1 High Performance Ion Exchange Chromatography

High Performance Ion Exchange Chromatography [HPIEC] currently plays an important role in the separation and purification of peptides: cationic exchangers are used for neutral and basic peptides and anion exchangers are used for neutral and acidic peptides. The columns of HPIEC have two types of support materials, either totally organic materials or surface modified inorganic supports. Peptide separations have been achieved on either silica- based or polystyrene-divinlybenzene based microparticulate pourous ion-exchange stationary phases [67].

An important factor in ion- exchange chromatography is the support pore diameter, it greatly influences the separation of peptides on the column. The pore diameter must be sufficiently large enough to allow the penetration of large molecules as well as allow for maximun ion-exchange capacity. Temperature can effect peptide retention and selectivity as well, therefore optimal temperature will vary for different peptide mixtures [68]. The resolving power of an ion-exchange column is also affected by ionic strenth and pH. Cachia [69] found that a mixture of peptides which vary with respect of net charge and basic residue composition could be separated on a carboxymethyl cellulose [CM] 300 weak cation- exchange column. The number of ionizable groups present on the CM supports were increased with increasing the pH [i.e. 4.5 to 6.5], thus increasing the resolving power, but at the same time more than doubled the time necessary to complete the separation. Also, an increase in solutes, such as potassium chloride, decreases the retention time of proteins but the separation between structurally related and similarly charged peptidesis is maintained.

Elution of a peptide bound to an ion-exchange column can be achieved by changing the ionic strength or the pH of the mobile phase [70]. Peptides have been successfully separated on HPIEC using mixtures of

acetonitrile and triethylammonium acetate [TEAA] buffer as the eluent [71]. This buffer system had minimal absorbance in the low UV allowing detection of the peptides in the 210-225 nm range. Also the TEAA is a volatile buffer, therefore the buffer can be removed during lyophilization making the system useful for preparative chromatography and subsequent direct bioassay. Other than UV absorption, peptides eluting from the columns can be detected by a variety of other methods including: fluorescence methods [72], differential refractometer [73], radioactivity detectors [73] and ninhydrin based monitoring systems [74].

One advantage of HPIEC is that column length has minimal influence on resolution [75] therefore protein samples may be eluted in a smaller volume of mobile phase and detector sensitivity will be greater [70]. Also speed of enzyme recovery on a smaller column is important when attempting to purify labile proteins [70].

Applications of HPIEC for peptide separations include: separation of proteolytic digests [76,77]; analytical peptide mapping [78,79]; analysis of peptides hormones [80,81] and the detection of abnormal hemoglobins in the newborn [82].

4.2 High Performance Size Exclusion Chromatography

Many investigators have found that size exclusion chromatography [SEC] or gel permeation high performance chromatogaphy [GP-HPC] yields a linear relationship between void volume [i.e. retention time] and log molecular weight of a peptide [1,70,83]. Furthermore because peptides of the same molecular weight vary in molecular shape due to functional groups along the side chains, and hydrodynamic volume, retention in SEC is also shape dependent. Thus, calibration curves cannot determine the molecular weight of a peptide of unknown shape with any degree of certainty. Therefore it is important that the mobile phase of SEC must be one that will eliminate partitioning and/or absorption on the packing material.

Rivier [83] points out that without addition of a detergent, adjustment of the pH and the ionic strength of the eluent is necessary for the separation of peptides according to size. A linear relationship was found between log molecular weight [1,000 - 44,000] versus retention time when using a Pac I-125 [Water Associates] in the presence of triethylammonium phosphate or formate buffer [pH < 3] in the presence of 15-30 % acetonitrile. This HPGPC system required a noncompressible support with minimal non-specific adsorption and controlled pore size. The advantage of this system include biological compatibility, low ionic strength and UV transparency.

Richter and coworkers [84] also developed a procedure for molecular weigth determination of peptides using a Bio-Sil Tsk 20 and Bio-Gel TSK 125 columns. In the presence of 6M guanidine hydrochloride, standards from 1000 to 10,000 were inversely correlated to the logarithms of their molecular weight on the TSK 20 columns. Two combined TSK 125 columns gave reliable separation of peptides in the molecular weight range from 800 to 3500.

Combined gel permeation HPLC and reverse-phase HPLC followed by radioiminoassay have been used to separate and quantitate beta endorphin related pepides [2]. Initially, the peptides are separted by molecular weight on gel permeation HPLC under denaturing conditions [6M guanidine HCl/0.2 M triethylamine phosphate]. This system provided accurate molecular weight deteminations for proteins of 500 - 20,000 daltons. Since larger proteins eluted in the void volume, it is not possiblle to determine their molecular weights. These beta endorphin related peptides were then analyzed by reverse-phase HPLC employing acetonitrile as the organic modifier and either trifluoroacetic acid [TFA] or heptafluorobutyric acid [HFBA] as the ion pairing agent. Separation of the peptides by reverse-phase HPLC revealed small differences in their structures resulting from minor post-translational modifications , such as acetylation, which affect peptide hydrohobicity. A combination of gel permeation and reverse phase HPLC were also used by Anderson and Mole [85] to isolate chemical and enzymatic cleavage fragments of plasma amyloid P-component for amino

acid sequence analysis.

4.3 Reverse-phase High Performance Liquid Chromatography

Peptides and protein of molecular weight less than 12,000 can be separated and eluted from reverse phase columns under the appropriate conditions of buffer composition, pH and organic modifiers. Reverse phase simply means that the molbile phase is more polar than the effective stationary phase. These reversed phase chromatography columns are prepared by binding alkyl residues to inorganic support materials; both octyl and octyldecyl-bonded as well as alkylphenyl, phenyl, and cyanoalkyl phases have been used successfully for peptide separations [70,86]. The chemical composition of the stationary phase is a function of unreacted surface available to the mobile phase components. Recently, Tempst and coworkers [87] found cyanopropylsilyl columns are able to separate peptides ranging in size from 5 amino acids to 68 kilodaltons.

The elution of peptides from the column requires an organic solvent which must be an effective solvent for the peptides of interest. If the peptides are not soluble in the chosen solvent, they may then precipitate in the column. The starting solvents [acid-basic buffers] that have been used to separate peptides by reverse-phase HPLC are dilute phosphoric acid [88], high concentrations of acetic acid or formic acid [89] and low concentrations of perflourinated organic acids, particularly triflouroacetic acid [90]. The main limiting solvents used in these systems include 1-propanol, 2-propanol, ethanol, methanol, or acetonitrile. Hermodson suggested separating small and hydrohilic peptides with methanol or acetonitrile gradients and larger hydrophobic peptides with propanol gradients [91]. All of these solvents allow spectrophotometric detection of the peptide bond in the range of 200 to 220 nm , hence allowing monitoring at high sensitivity.

Other than insolubility of the peptide in the organic solvents, these solvents do present other problems. For example, nonvolatile solvents such as phosphoric acid would be difficult to remove from the peptide

mixture. Also, the high viscosity of some of the solvents such as propanol may limit resolution but usually has no effect on peptide recovery [91]. And finally, since these peptides are partially denatured in the solvents therefore resulting in loss of biological activity, separation of enzymes on these columns may prove to be difficult.

In the presence of strongly ultraviolet light absorbing solvents such as pyridine, nanogram levels of peptides can be detected by postcolumn derivitization of free amino groups with fluorescamine [92] or the epsilon amino group of lysine with O-phthaladehyde [93]. Meek has also shown that derivatizing peptides with 3,6 dinitrophthalic anhydride and 2- carboxy-4,6-dinitrophthalic anhydride allows analysis of peptides in tissue extracts that are in too low concentrations [< 100 pmol] to be detected by absorbance at 210 nm [94].

The pore size of a reverse-phase HPLC column dramatically affects the behavior of the peptides on the column [95]. If peptides less than 25 residues are to be resolved there are high density columns available with small pore sizes [97] that will adequately resolve these small peptides. Many researchers claim that neither flow rate, length of the column, nor temperature [96] has a dramatic effect on the resolution of peptides on reverse phase HPLC. Rivier [97] claims higher temperatures resulted in lower resolution but other investigators claim temperatures of 45°C result in better separations [98] or where the effect of temperature is of minor importance [99]. Although sample loading does affect retention of peptides with alkylsilicas, overloading does not result in total loss of resolution as may occur in size exclusion of ion-exchange chromatography [100].

Peptides tend to give better resolution on alkylsilicas for solvent pH values in the range of 2-3 [100]. When choosing a solvent pH, an investigator must take into account the possibility that a specific protein will be insoluble at or near its pI value.
An advantage of reverse-phase HPLC is that very closely related molecules can be separated using combinations of pH and pairing ion

modulation under specific gradient elution conditions [88,100,101]. Proper choice of a charged counter ion depends on the composition and sequence of the peptide [102]. Hydrophobic ion-pairing reagents can be used to significantly increase retention of polar peptides whereas hydrophilic ion pairs increase the polarity of the peptide resulting in a decrease in its retention on a reversed-phase column. This technique offers many other advantages: the chromatography is fast; detection of the peptide bond at 210 nm is quantitative and highly sensitive; and recovery of the peptides loaded on the column is complete.

The major application of revers-phase HPLC is the isolation and purification of peptides [103]. Other than giving an indication of the hydrophobicity of the peptides ,limited information about the peptide is obtained by this method. In addition, reverse phase HPLC has been used as a procedure to detect protein variants [104,105,106,107]; for analytical peptide mapping [108,109]; as well as for analysis of synthetic peptides produced by solid-phase or solution peptide synthesis [110]. Reverse-phase HPLC has also facilitated structural studies of glycoproteins [111].

5 conclusion

Paper chromatography and electrophoresis were among the first methods used to separate peptides from complex mixtures. Thin layer techniques were latter adapted for their greater capacity, and preparative potential. Both the paper and thin layer techniques have proven to be useful in qualitative analysis but limited in their quantitative abilities. They are also unable to separate larger peptides, peptides which are insoluble in the required buffers, and samples which contain high concentrations of inorganic ions. At present time polyacrylamide gel electrophoresis is limited in its ability to deal with mixtures containing peptides of widely ranging sizes and the small peptides. The two main problems with this technique are: co-electrophoresis of small peptides and limitations in the ability to detect the small peptides,

due to diffusion and loss of the peptides from the gel. High performance liquid chromatography, a popular method of choice for studying peptides offers the capability of: estimating of the molecular weight of small peptides, through the use of size exclusion chromatography; information on the charge of the peptides, by the use of ion exchange chromatography; and a measure of peptide hydrophobicity may be determined by reverse-phase chromatography. Although all these procedures allow us to study unique problems of peptides, there is not yet available a general analytical technique for the separation and detection of peptides with widely varying molecular weights, particularly for the small molecular weight peptides containing less than 10 amino acid residues.

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6 References

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VIRUSES AND VIRAL COMPONENTS IN EXTRACTS OF INFECTED CELLS: ANALYSIS BY AGAROSE GEL ELECTROPHORESIS

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Summary

Detection, isolation, chracterization and quantitation of viruses and related particles are goals of diagnostic and basic virology. Progress in the use of agarose gel electrophoresis to accomplish these goals is described here. This technique is comparatively inexpensive and nonselective for the detection of particles in either unfractionated or partially-fractionated lysates of infected cells. The size of spherical particles has been measured with an error of ± 1% by two-dimensional agarose gel electrophoresis. Spheres can be discriminated from rods by measurement of electrophoretic mobility (μ) as a function of gel concentration. Values of solid support-free μ can be measured. Agarose gel electrophoresis is used in the analysis of bacteriophage assembly pathways and has potential for use in analysis and diagnosis of virus-caused diseases.

1. Introduction

Detection and characterization of viruses and subviral, virus-related particles are objectives of diagnostic virology (reviewed in 1-3) and basic virology (reviewed in 4-6). Detection has been performed by testing for particles with either the known biological, biochemical or immunological properties of known viruses and virus-related particles. However, specific biological, biochemical and immunological properties, in contrast to physical properties, are not a characteristic of all virus-like particles. Therefore, testing for particles with known biological, biochemical and immunological properties will be referred to as intrinsically selective. To eliminate intrinsic selectivity, detection has been performed by testing for particles with virus-like physical properties. For example, in clinical virology, electron microscopy has been used to detect viruses (7-9). In basic virology, viruses and viral precursors have been detected by electron microscopy, rate zonal centrifugation, buoyant density centrifugation and gel electrophoresis (reviewed in 4-7,10,11). Although there is no intrinsic selectivity in the use of such physical procedures, these procedures can be selective (extrinsic selectivity) because of: a) alteration of the particles by the procedure used for their analysis (for example, either disintegration of particles or adherence of particles to solid supports), b) failure of the procedure of analysis to discriminate two related, but different, particles.

Gel electrophoresis is 10-100 times less expensive (in time and cost) than the other physical procedures above. Procedures for measuring electrophoretic mobility (μ) $\pm$ 2% in agarose gels have

been developed (12). Therefore, it appears worthwhile to explore the possibilities for use of agarose gel electrophoresis in basic and clinical virology. Agarose gels with pore exclusion limits as large as 400 nm and as small as 10 nm in radius can be used for electrophoresis (P. Serwer and S. J. Hayes, unpublished data). Therefore, pore size is not a limit for the analysis of known viruses.

2. Adherence to the Gel

In some cases, adherence to agarose has limited the use of agarose gel electrophoresis for the detection and characterization of viruses and virus-related particles. Adherence has been revealed by: a) an increase in the sharpness of bands as the concentration of sample is increased (13), and b) a decrease in the $|\mu|$ to 0 as time of electrophoresis is increased (14). Bacteriophage T7, but not the genetically related bacteriophage T3, adheres detectably to agarose during electrophoresis. Fibers on an external tail of T7 are required for both attachment to the bacterial host and adherence to agarose (13,15). Although other double-stranded DNA bacteriophages have tail fibers (16), only T7 among ϕ29, P22, T3, T4 and T5 adheres detectably to agarose during electrophoresis (reviewed in [6]). A variety of other bacterial, plant and animal viruses also appear not to adhere to agarose during electrophoresis (reviewed in [6]). At present, the structure of viral components is not well enough known to understand the cause and predict the occurrence of such adherence.

Agarose is a mixture of related polymers and characteristics of agarose gels can vary with the source and preparation of agarose. In addition, the properties of agarose gels can be modified by either covalently modifying agarose or by adding other polymers to agarose

before gelation (reviewed in [17]). Thus, if a particle adheres to gels made from one preparation of agarose, it appears worthwhile to test additional preparations in order to find a preparation that is nonadherent. One possible procedure for performing this test has previously been described (13). Most commercially-prepared agarose and mixtures containing agarose are sold with a manufacturer-determined index of electro-osmosis (EEO), the electrophoresis-induced movement of buffer through the gel (18,19). EEO increases as the concentration of anionic groups on agarose polymers increases (20). As agarose is increasingly used for the electrophoresis of proteins and viruses, it is hoped that eventually assays of the protein binding of agarose gels will be developed and the results of such assays will be provided by the manufacturer of commercially-produced agarose.

3. Detection of Particles

Viruses consist of nucleic acid packaged within a container (capsid) of protein that in some viruses is covered by pieces of cellular membrane (21). Because the nucleic acid is almost always resistant to nucleases, agarose gel electrophoresis of nuclease-treated samples, followed by staining for nucleic acid and for protein, is a comparatively non-selective procedure for detecting either viruses or virus-related nucleoproteins. Ethidium bromide has been used to stain DNA packaged in bacteriophages, without staining protein (13,22). The limits of detection were 2 ng of DNA (= 1.0×10^8 particles of bacteriophage ϕ29, molecular weight of DNA = 12×10^6 or 1.0×10^7 particles of bacteriophage T4, molecular weight of DNA = 115×10^6). The data (23) suggest that improved sensitivity of DNA-specific staining is possible. A systematic attempt to increase the staining sensitivity for nucleic acids in agarose gels should be made.

Agarose gel electrophoresis, followed by staining with ethidium bromide, has been used to detect viruses in unfractionated extracts of cells infected by a tail fiberless mutant of bacteriophage T7 (24), panicum mosaic virus (a plant virus that infects grasses) and viruses related to panicum mosaic virus (25). This procedure should be applicable for diagnosis of the diseases of both plants and animals. Among possible limitations of the above procedure are: a) sensitivity, b) possible adherence of viruses to agarose gels (see the Discussion above), and c) interference by cellular components. Thus far, interference by cellular components has not been a limitation. A procedure for assisting in isolating viruses from cellular components during agarose gel electrophoresis is described below.

4. Characterization of Particles

4.1. Staining

In addition to its use for detection in agarose gels, nucleic acid-specific staining can also be used to characterize the internal contents of viruses and related particles after fractionation by agarose gel electrophoresis. For instance, a staining procedure for detecting bacteriophage capsids with subgenomic, packaged DNA was developed and used for bacteriophage P22 (26). Because of their comparatively small amount, the finding of such particles was like finding a needle in a haystack. By staining of the viral nucleic acid with acridine dyes it should also be possible to distinguish DNA-containing from RNA-containing viruses after electrophoresis (27).

4.2 Electron Microscopy

To help characterize particles after detection by agarose gel electrophoresis of either unfractionated or partially fractionated

extracts, these particles are eluted from the gel and examined by electron microscopy. For instance, in the case of tobacco mosaic virus (TMV), a rod-shaped plant virus with a length of 300 nm and a radius of 9 nm (28), two sharp bands, and possibly a third, were observed by Coomassie blue staining of agarose gels after electrophoresis of a partially fractionated extract of infected Hicks tobacco leaves (Fig. 1). These bands can also be seen, without significant loss in clarity, in profiles of unfractionated extracts less than two weeks old (not shown). To determine the characteristics of the particle that forms each of these bands, the following procedure (not previously described) was used to transfer particles directly from the gel to a support film for electron microscopy.

Two identical samples were separately subjected to electrophoresis in a 0.9% agarose slab as shown in Figure 1. The region of the gel traversed by one of the two samples was excised and stained with Coomassie blue. Using this stained section of the gel as a template, the position of the above bands in the unstained section of the gel was determined. A groove was cut through the estimated center of each band, using either a spatula or a razor knife. Into each groove was placed a 400 mesh, copper electron microscope grid, covered with a carbon film. The carbon film had been placed on the grid, as previously described (29), and faced the cathode. To help deposit particles on the carbon film, electrophoresis at 1 V/cm was then performed for 2 hr with the grids in the gel. The grids were then removed from the gel and negatively stained with uranyl acetate, as previously described (29). To determine whether or not the grids had been accurately placed for elution, the gel used for elution was subsequently stained with Coomassie blue.

Rods with the radius and central hole of TMV were observed when particles eluted from the region of the gel containing band 2 of Figure 1 were observed (Fig. 2). However, 34 of 52 particles observed were too long to be monomers and had the length of an end-to-end TMV dimer. A gap at the point of joining between monomers was observed at the mid-point of some dimers (arrowhead in Fig. 2). By a similar analysis, the band labeled 3 in Fig. 1 is formed by an end-to-end trimer of TMV. A band potentially formed by TMV monomers is indicated with a question mark in Figure 1. End-to-end multimerization of TMV has previously been observed by rate zonal centrifugation (30).

4.3 Sieving during unidirectional electrophoresis

The electrophoretic mobility (μ) of a particle in an agarose gel is determined by the: a) μ in the absence of the gel (μ_o) and b) reduction of $|\mu|$ caused by collision of the particle with the gel's network of fibres (sieving). For virus-sized (13-42 nm in radius) spherical particles in gels with an agarose percentage ($\underline{A}$) ≤ 0.9, the following relationship has been found (reviewed in [17]):

$$\mu = (\mu_o + \mu_E)_e^{-K_R \cdot \underline{A}} \qquad (1)$$

μ_E describes the EEO and K_R is the empirically-obtained slope of a semilogarithmic μ vs. $\underline{A}$ plot. K_R increases as the radius (R) increases (17). For $\underline{A} \geq 0.9$, semilogarithmic μ vs. $\underline{A}$ plots have significant nonlinearity and K_R progressively increases as $\underline{A}$ increases (convex curvature).

In the presence of spherical size standards of known R, the R of a spherical particle can be determined from the K_R with an accuracy of ± 8% (reviewed in [17]). By plotting μ vs. $\underline{A}$ (instead of ln $|\mu|$ vs. $\underline{A}$) it has been found for at least one 6.5% hydroxyethylated agarose

that $\underline{A}$ extrapolated to a μ of 0 ($\underline{A}_o$) is independent of μ_o and decreases as R increases (31). From $\underline{A}_o$, R can be determined with an accuracy of ± 4% (31).

Semilogarithmic μ vs. $\underline{A}$ plots for rod-shaped viruses are linear for $\underline{A}$ below a critical value that decreases as the length of the rod increases (32). Above this critical $\underline{A}$ the plot becomes concave (32), in contrast to the convex curvature observed for spheres (above). Thus, a rod-shaped virus can be discriminated from a spherical virus by semilogarithmic μ vs. $\underline{A}$ plots. Additional studies are needed to determine how accurately the shape of a particle can be determined by measurement of its sieving in agarose gels, without additional information.

4.4 Two-dimensional electrophoresis

After a single, unidirectional electrophoresis, the position of a particle in a gel is a function of two independent properties of the particle, μ_o and a size-shape-describing parameter (K_R or R for a sphere, if $\underline{A} \leq 0.9$). Position in the gel does not uniquely determine μ_o and R. To obtain a gel profile with a one-to-one correspondence between position in the gel and the R of a spherical particle, the following procedure of electrophoresis in two directions (two-dimensional agarose gel electrophoresis) was developed.

A first electrophoresis is performed in a comparatively dilute agarose gel (first-dimension gel). Subsequently, particles fractionated in the first-dimension gel are subjected to electrophoresis orthogonal to the first-dimension gel and migrate into a more concentrated gel (second-dimension gel) adjacent to the first dimension gel. As previously shown (33), two-dimensional agarose gel electrophoresis distributes

all spherical particles of any one R along a straight line (size line) that passes through the effective origin of electrophoresis and makes an angle, θ, with the first-dimension gel. As R decreases, θ increases and R(θ) is determined with size standards (33). If the A of the first-dimension gel is low enough so that its sieving is too low to be detected, then the μ_o' of a particle after two-dimensional agarose gel electrophoresis is μ in the first-dimension gel.

Because the position of a spherical particle after two-dimensional agarose gel electrophoresis uniquely determines R and μ_o, the patterns obtained by this procedure can, in theory, be used to determine the distribution of R and μ_o for heterogeneous particles in a mixture. The automated densitometry and computer software for achieving this goal has not yet been developed. The sorting by size that occurs during two-dimensional agarose gel electrophoresis should also help separate viruses from interfering subcellular fragments of cells, during the fractionation of crude extracts of virus-infected cells. By adjusting the A of the second-dimension gel, resolution of R as good as ± 0.3 nm has been achieved for particles approximately 30 nm in R (33). Thus, in the presence of standards of known R, R can be measured ± 1% by two-dimensional agarose gel electrophoresis.

5. Analysis of Viral Assembly Pathways

When analyzing the assembly pathway of a virus, one first tries to detect particles (called assembly intermediates) in the pathway. After detecting potential assembly intermediates, their kinetics of appearance is determined (examples are in 4-6, 34). Determination of these kinetics requires quantitation of potential intermediates in multiple samples. Performing this quantitation by agarose gel electrophoresis, instead of either centrifugation or electron microscopy,

converts work requiring several months into work that can be (and has been) completed in a week (a comparatively recent example is in [34]). The data obtained are also usually more accurate than the data obtained by centrifugation.

If agarose gel electrophoresis is not, by itself, sufficient to fractionate all potential assembly intermediates, agarose gel electrophoresis can be used as the second step in a two-step procedure of detection and quantitation (6). The first step can be centrifugation (either rate zonal or buoyant density), electrophoresis (either density gradient or gel; see above), or column chromatography.

6. Determining the Cause of Infectious Disease: Analogy with Determining of Viral Assembly Pathways

If the virus that causes an infectious disease is the most abundant virus in extracts of infected tissue, it is comparatively easy to identify this virus. However, if other, non-causative viruses or virus-like particles (secondary invaders, for instance) are more abundant than the particle that causes the disease, identification of the particle that causes the disease becomes more difficult. The difficulties are compounded if the virus has not been biologically cloned and propagated so that Koch's second and third postulates can't be used. Finding a disease-causing particle, in the presence of a larger amount of other particles, is analogous to the finding of an assembly intermediate that is present in comparatively small amounts. The success of agarose gel electrophoresis-ethidium staining in this latter pursuit (above) suggests that it is worthwhile to use this procedure in attempts to detect, isolate and characterize disease-causing viruses.

If a potential disease-causing particle is detected by agarose gel electrophoresis, comparison of the particle's appearance kinetics with the kinetics of disease symptoms is a procedure to help determine the relationship of symptoms to the presence of the particle. Experimentally, the particle's appearance kinetics could be determined by agarose gel electrophoresis, as performed for determining the appearance kinetics of assembly intermediates (above). Extending the analogy with assembly even further, it is possible that some diseases of currently unknown cause are caused by more than one virus, the different viruses appearing in a definable temporal order. Instead of determining the single virus that causes a disease, in this case one would determine the viral pathway of causation. For example, evidence of a bacterial pathway of causation has been presented for early-onset periodontitis (35).

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Figure 1. Agarose gel electrophoresis of an unfractionated extract of leaves infected by TMV. Thirty g of Hicks tobacco leaves infected with the common strain of TMV, received from M. Langham and R.W. Toler, Texas A & M University, were mixed with 100 ml of ice-cold 0.05 M sodium phosphate, pH 7.4, 0.001 M $MgCl_2$ (electrophoresis buffer) and were subsequently ground in a Waring blender for 5 min. This extract of ground leaves was clarified by two successive centrifugations at 10,000 rpm, 4°C for 10 min in a Beckman JA-14 rotor. Particles of TMV were pelleted by centrifugation at 18,000 rpm, 4°C for 16 hr. in a Beckman JA-20 rotor. After resuspending the pellet in a 1/100X volume of electrophoresis buffer, the preparation of virus was clarified once more, as described above. To 2 µl this crude preparation of TMV was added: 30 µl of electrophoresis buffer; 1 µl of a preparation containing 3 µg of the spherical RNA bacteriophage, MS-2 (used as a marker for µ); and 62 µl of 4% sucrose, 400 µg/ml bromphenol blue in electrophoresis buffer. Of this mixture, 50 µl was subjected to electrophoresis in a 0.9% agarose gel (Seakem ME agarose, purchased from the Marine Colloids Division of the FMC Corporation) at 1 V/cm, room temperature (25 ± 3°C) for 16 hr. The 0.9% gel was embedded in a 1.5% agarose frame, as described in ref. 19. After electrophoresis, protein in the gel was stained with Coomassie blue (19). The origin of electrophoresis is indicated by the arrowheads; the direction of electrophoresis (toward the anode) is indicated by the arrow. Bands determined by electron microscopy (see the text) to be those of TMV dimers and trimers are indicated by 2 and 3, respectively. The band formed by bacteriophage MS-2 is indicated.

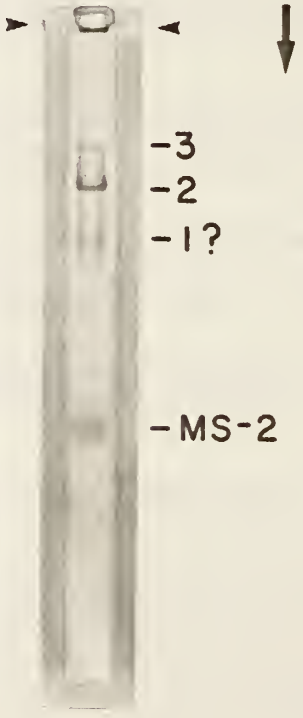
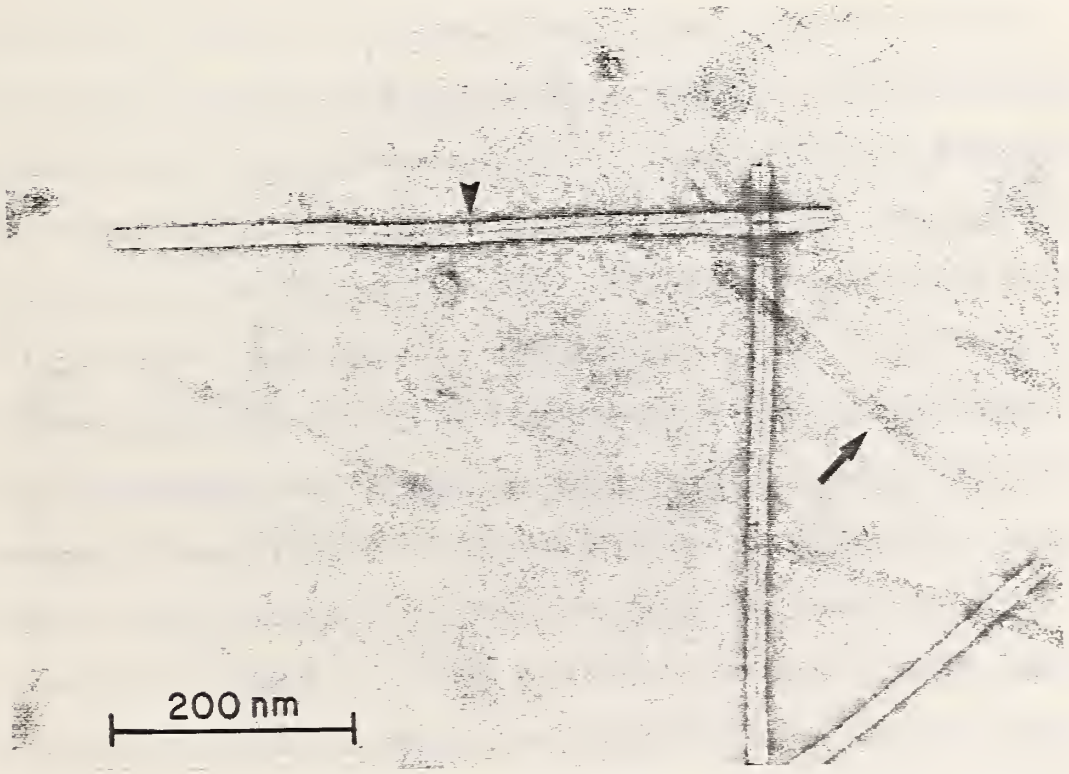


Figure 2. Electron microscopy of a dimer of TMV. Particles from the region of an agarose gel indicated by 2 in Fig. 1 were eluted from the gel and prepared by electron microscopy, as described in the text. An example of a dimeric TMV rod is shown; the point of joining between the two monomers of the dimer is indicated by the arrowhead. The arrow indicates a monomeric TMV rod.



Gel Electrophoresis of DNA

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Recent studies of the electrophoresis of DNA in agarose and in polyacrylamide gels are summarized. For agarose gel electrophoresis, the topics covered include: quantitative studies of the electrophoresis of linear restriction fragments and spherical DNA molecules; the use of pulsed electric fields; the separation of chromosome-sized molecules of DNA using alternating inhomogeneous electric fields; the application of electric birefringence to the study of gel electrophoresis; recent developments in reptation theory; the use of gel electrophoresis to study conformational transitions; the pore size and fiber radius of various types of agarose; and recent advances in experimental techniques. For polyacrylamide gel electrophoresis, the topics covered include: quantitative studies of the electrophoresis of linear restriction fragments; the anomalous mobility exhibited by some restriction fragments; the use of denaturing gradient gels to study single base mutations; the study of protein-DNA interactions by a gel electrophoresis assay; and improvements in experimental procedures.

1 Introduction

Electrophoresis is the movement of charged particles suspended in a medium under the influence of an electric field (1, 2). In free solution, the electrophoretic mobility of DNA molecules is independent of molecular weight (3), because the linear charge density is constant for all molecules. However, when electrophoresis takes place in a supporting gel medium, molecular weight separation occurs. Various applications of gel electrophoresis range from the separation of DNA fragments containing a few base pairs for sequence analysis, to the separation of whole chromosomes, containing hundreds of thousands of base pairs (4).

Despite the importance of gel electrophoresis in the development of modern molecular biology, the actual mechanism of the molecular weight separation of DNA fragments in the gel is not clear (5, 6). Two different mechanisms have been proposed. The first, based on the Ogston model of a random meshwork of fibers (7), hypothesizes that the electrophoretic mobility of a macromolecule is proportional to the volume fraction of the pores of a gel that it can enter (5, 8-10). Since the average pore size decreases with increasing gel concentration, (5, 8-11), electrophoretic mobility is expected to decrease with increasing gel concentration. The second mechanism is based on reptation, the "worm-like" or "snake-like", end-on migration of DNA molecules through the pores of gel (12-14). This mechanism has been used to explain the inverse relationship between electrophoretic mobility and DNA molecular weight observed experimentally (15).

In this review, recent experiments utilizing gel electrophoresis will be summarized. The emphasis will be on illustrating the different types of experiments for which gel electrophoresis is a useful technique, rather than an exhaustive survey of the literature. Two types of gel matrices are commonly used for the gel electrophoresis of DNA: agarose and polyacrylamide. Since different types of experiments are carried out in the two media, they will be presented separately. Some practical aspects of gel electrophoresis are discussed in (16-18).

2 Agarose gel electrophoresis

2.1 Background

The chemistry of agarose and the physical properties of agarose gels have been reviewed by Serwer (19). Solid agarose is dissolved by heating in an appropriate buffer, and undergoes gelation upon cooling. Gelation in aqueous solution is accompanied by the formation of aggregates of double-stranded helices (20, 21). Gels formed in the presence of salt have larger void spaces and a stronger tendency for sections of rods to group together (21). Aqueous solutions of agarose appear to undergo a sol-sol transition as the first step in gelation (22). Under conditions where gelation occurs over a period of weeks to months, static and dynamic light scattering experiments indicate the presence of a transition in the sol state which occurs after a characteristic incubation time (22). This transition may reflect the coil-to-helix transition preceding gelation (22).

2.2 Linear DNA restriction fragments

2.2.a Relation between mobility and molecular weight

Several recent studies have focused on the quantitative relationship between gel electrophoretic mobility and DNA molecular weight. For high molecular weight fragments, these curves depend on experimental parameters such as gel concentration and electric field strength (6, 23). Southern (15) proposed that the reciprocal of electrophoretic mobility is linearly related to molecular weight. Bearden (24) proposed that gel electrophoretic mobilities are related to $M^{-2/3}$, over a restricted range of experimental conditions. Stellwagen (25) proposed that mobilities of restriction fragments containing fewer than 1000 base pairs (bp) are linearly correlated with $M^{-0.8}$, because of the variation of the "hydrodynamic surface area" of fragments of this size with molecular weight. Edmondson and Gary (26) proposed that gel mobilities are related to $[M_1(L_1/L_o)^{-x} - M_2]$, where M_1 and L_o are constants and x and M_2 depend on the gel concentration.

2.2.b Determination of K_R

The negative slope of the natural logarithm of the apparent mobility as a function of gel concentration is called the retardation coefficient (5, 8-10). A semi-logarithmic plot of the apparent mobility as a function of gel concentration is linear for DNA restriction fragments up to about 1-3 kilobase pairs (kbp) in size (5, 6, 24-28). For

larger fragments such plots show a concave upward curvature (6, 24, 25, 27, 28). The degree of curvature depends on the DNA molecular weight and applied voltage, and can be essentially eliminated by extrapolating the mobilities observed at each gel concentration to zero electric field strength (6). These effects are due to the fact that the mobilities of restriction fragments in agarose gels increase linearly with increasing electric field strength; the rate of increase decreases with increasing molecular weight (6). It has been suggested that high electric fields may not give the particles enough time to accelerate to their free solution electrophoretic velocities before colliding with a gel fiber (29).

According to pore size distribution theory (5, 8-10), the retardation coefficients can be related to molecular parameters by

$$K_R^{1/2} = a + b(R+r) \qquad (1)$$

where a and b are empirically determined constants, R is the radius of a spherical particle moving through the gel, and r is the gel fiber radius. For highly asymmetric particles, such as small DNA restriction fragments, the correct value to use for R has been a matter of debate (5). Bearden (24) used a prolate ellipsoid of revolution with constant axial ratio. Stellwagen used the hydrodynamic surface area determined from electric birefringence experiments for restriction fragments $\leq$ 1 kbp (25), and the root-mean-square (rms) radius of gyration for larger fragments (6). Serwer and Allen (28) used an effective hydrodynamic radius calculated from the radius of gyration. Edmonson and Gray (26) approximated the dependence of K_R on macro-

molecular length by a logarithmic function. A log-log plot of K_R vs. molecular weight shows that $K_R \sim M^{0.6}$ for DNA fragments between 0.5 and 12 kbp in size, if the K_R values are determined from mobilities extrapolated to zero electric field strength (6).

2.2.c Apparent mobility at zero gel concentration

From semilogarithmic plots of mobility as a function of gel concentration, estimates can be made of the mobility of DNA fragments at zero gel concentration. This mobility should be close to the free solution value (8). A summary of the literature values of the mobility at zero gel concentration is given in Table I.

2.3 Spherical DNA particles

2.3.a Mobility as a function of molecular weight

In HGT agarose, (trademark name of Marine Colloids) open circle (relaxed) and closed circle (supercoiled) DNA molecules were found to have the same mobility at zero gel concentration as linear DNA molecules of the same molecular weight (28). Careful control of temperature and applied voltage were required to obtain this result (28).

A plot of the logarithm of the mobility of spherical bacteriophage particles as a function of gel concentration was found to be concave downward at high gel concentrations (28, 32, 33), opposite to the curvature observed with linear DNA molecules. In SeaPlaque agarose

(trademark name of Marine Colloids), the mobility of bacteriophage and related particles was found to be linearly dependent on gel concentration (34). The intercept of this plot with the axis of zero gel concentration was found to be related to the radius of the bacteriophage particle.

2.3.b Superhelix density

One application of agarose gel electrophoresis is the determination of superhelix density of closed circle DNA molecules. A series of topological isomers differing by one in linking number can be clearly resolved on agarose gels (35, 36). Typical applications have ranged from determining the helical periodicity of different conformations of DNA (37), to evaluating the dependence of conformational transitions on superhelix density (38).

2.3.c Two-dimensional electrophoresis

A variant of the method of determining superhelix density is running the electrophoresis in each of two perpendicular directions, using the same electric field strength in each direction. Before electrophoresis in the second direction, the gel is soaked in an intercalating agent (39). Topoisomers are resolved into distinct spots that lie along a smooth curve, each spot differing by one in linking number (39). An abrupt shift in the curve is an indication of a conformational transition. Current applications of this technique include determining helical periodicity (39), cruciform formation (40), and the in vivo occurrence of Z DNA (41).

Branched DNA molecules have been separated from linear molecules by electrophoresis in two perpendicular directions, using a different agarose concentrations and electric field strengths in each direction (42). The branched molecules appear on the gel diagonal, in order of increasing molecular weight.

2.4 Pulsed field electrophoresis

2.4.a One dimension

Instead of a continuously applied electric field, it is possible to use a pulsed square wave of varying amplitude and duration. The electrophoretic pattern can be significantly varied, even though the total energy (voltage x time) of the electric field is kept constant (43). The pulse amplitude, duration, and frequency can be varied to maximize the gel resolution in any given molecular weight range (43). Pulse amplitudes are typically a few V/cm, applied for a few seconds.

2.4.b Two dimensions, non-uniform fields

Schwartz and Cantor (44) have devised an electrophoresis procedure using pulsed, alternating electric fields in perpendicular directions to separate chromosome-sized molecules of DNA containing up to 2000 kbp. At least one of the perpendicular electric fields is inhomogeneous. The period and amplitudes of the applied pulses are varied to optimize the separation in a desired molecular weight range. Electrophoresis occurs on the diagonal of the gel (44). Another

experimental arrangement places the gel at a 45° angle to the mutually perpendicular electrodes, to give a nearly linear electrophoretic pattern in the gel (45). In these applications, the agarose gel concentration is typically 1.0-1.5%, with electric fields ∿3 V/cm. and switching times of 20-50 sec.

2.4.c Electric birefringence, single pulses

Electric birefringence uses refractive index differences to measure the orientation of DNA molecules, either imbedded in a gel or in free solution. The DNA molecules are oriented by a pulsed electric field; the applied voltage is on the order of 1 kV/cm., with durations in the millisecond range. The time constant for the loss of the orientation after removal of the electric field is characteristic of the size and shape of the macromolecule being oriented (46). Three DNA restriction fragments, ranging in size from 622 to 2936 bp were studied, imbedded in agarose gels ranging in concentration from 0.2% to 1.5%. The birefringence relaxation times of the three fragments were equal to their free solution values if the median pore diameter of the gel was greater than the effective hydrodynamic length of the fragment. However, if the median pore diameter was smaller than the effective hydrodynamic length, the birefringence relaxation times increased to values characteristic of fully stretched DNA molecules. The apparent elongation of the DNA molecules in the agarose gels indicates that reptation is a likely mechanism for the electophoresis of large DNA molecules (46). These results are summarized in Table II.

2.4.d Electric birefringence, reversing fields

If electric field pulses which are rapidly reversed in polarity are applied to DNA molecules imbedded in agarose gels, the birefringence (i.e., orientation) exhibits a transient at the moment of field reversal. The time interval between the moment of field reversal and the minimum in the orientation depends on pulse voltage, pulse length, and DNA molecular weight (47).

2.5 Reptation theory

Lumpkin, et al. (13, 14) have presented a theory for electrophoresis based on the axial movement, or reptation, of DNA molecules through "tubes" in the gel. The direction of the tube is random, except for possible bias introduced by the applied electric field. With small electric fields, an inverse length dependence of the mobility is found, as expected (48, 49). At higher fields the tubes become oriented, because the field biases the direction of the leading edge of the chain. This leads to an increase of mobility with increasing field strength by adding a field dependent but length-independent term to the mobility equation. Slater and Noolandi (50) have estimated that stretching of the DNA chains would take place early in the time scale of an electrophoresis experiment. The data of Bean and Hervet (56) and Stellwagen (6) can be described by the equations for reptation (14).

2.6 Structural transitions

The B/A transition in DNA has been studied by electrophoresis in agarose gels run in an ethanol gradient (51). DNA molecules at the mid-point of the transition appear to have the highest electrophoretic mobility, suggesting that the B/A junction is flexible (51).

A pH dependent structural transition in the homopurine-homopyrimidine tract in superhelical DNA has been studied by two-dimensional electrophoresis (52). A new structural conformation, based on a cruciform structure, has been suggested (52).

2.7 Pore size and fiber radius

Three estimates have been given for the pore sizes of agarose gels of different types. Righetti et al. (53) used polystyrene latex particles of known diameter to determine the limiting pore size of Isogel agarose (trademark of Marine Colloids) gels of different concentration. The equation relating these two parameters is

$$P = 140.7\ c^{-0.7} \qquad (2)$$

where P is the pore diameter in nm and c is the gel concentration in percent (presumably the hydrated gel powder). Serwer and Allen (34) studied the electrophoresis of spherical virus particles of known molecular radius, and found the equation

$$P = 117.4\ c^{-1.71} \qquad (3)$$

for SeaPlaque agarose (trademark name of Marine Colloids). Stellwagen (6) determined the retardation coefficients of a series of high molecular weight DNA restriction fragments in LE agarose (trademark name of Marine Colloids), and used the root-mean-square radius of gyration to estimate the median pore diameter:

$$P = 25 + 70\ c^{-1.0} \qquad (4)$$

From these three equations, the median pore diameter of a 1% gel would be 282 nm for Isogel agarose, 234 nm for SeaPlaque agarose, and 190 nm for LE agarose. The decreases in estimated pore size correlate with the sieving order determined by Serwer, et al. (33) for these three types of agarose.

The gel fiber radius of various types of agarose was determined by Serwer, et al. (33) from plots of $K_R^{1/2}$ vs. the radius of spherical bacteriophage particles. The fiber radii were found to be 25 nm for HGT, ME, SeaPlaque and SeaPrep agarose, and 20 nm for Isogel agarose (all trademark names of Marine Colloids).

2.8 Experimental improvements

Serwer (54) has described an apparatus and techniques for running several agarose gels of different concentration simultaneously. Gels as low as 0.035% in concentration have been formed and run (55). Improved procedures of controlling voltage gradient and temperature have been described (56). Multiple gels containing ethidium bromide of different concentrations in different lanes have been used to measure superhelix density in horizontal gels (57).

The electric field dependence of the apparent mobility of DNA restriction fragments has been the subject of several studies (6, 23, 25, 28, 58).

The effect of electroendosmosis on the apparent mobility observed in different types of agarose has been discussed (6, 19, 59, 60).

An apparatus for two dimensional gel electrophoresis without gel manipulation has been described (61).

Methods of quantifying the fluorescence of ethidium bromide-stained horizontal gels have been developed for one-dimensional (62) and two-dimensional (63) electrophoresis. Quantitative measurements of fluorescently stained DNA from photographic images have been described (64). A computer-automated method of evaluating one-dimensional gel electrophoretic radiograms has been described (65).

The resolution of DNA fragments ranging from 30 bp to 15 kbp in a single agarose gel has been achieved by pouring a slab gel of increasing thickness from cathode to anode (66).

The effect of DNA-agarose binding affinity has been studied for several types of agarose (67). Forward fronting of the bands at high DNA concentrations and "displacement" by carrier DNA leading to band sharpening are interpreted in terms of DNA-agarose interactions (67).

3 Polyacrylamide gel electrophoresis

3.1 Background

Polyacrylamide gels are formed by the polymerization of acrylamide and a cross-linking agent, such as N,N'methylene bisacrylamide (Bis). The extent of the reaction and the rate are governed by the concentration of initiator (18). Control of the polymerization reaction depends on reagent purity and stoichiometry (68). Small angle x-ray, neutron, and light scattering studies (69) indicate that 8% polyacrylamide gels polymerized with 0-0.5% Bis contain two phases, a homogeneous phase of constant polymer content, with fiber radius increasing with increasing Bis concentration, and an inhomogeneous phase, characterized by large concentration fluctuations. The viscoelastic properties of 2.5% and 5% polyacrylamide gels have been measured by polarized light scattering (70).

3.2 Linear DNA restriction fragments.

3.2.a Relation between mobility and molecular weight

The polyacrylamide gel electrophoresis of linear DNA restriction fragments between 30 and 4000 bp in size has been studied by Stellwagen (71). The electric field dependence of the apparent mobilities of fragments in this size range is small (71, 72) and virtually independent of molecular weight (71). However, for DNA molecules greater than about 10 kbp in size, the apparent mobilties have been found to de-

crease abruptly at low electric field strengths in gels containing 1.0-1.5% polyacrylamide and 0.5% agarose (73).

3.2.b Retardation coefficients

Stellwagen (71) determined the mobility of a series of linear restriction fragments in polyacrylamide gels of different concentrations. The apparent mobilities observed for small DNA fragments, extrapolated to zero gel concentration, ranged up to 3.4×10^{-4} cm^2/V sec (71). These values are higher than the free solution mobility of 1.85×10^{-4} cm^2/V sec (3), possibly because of specific interactions between the aromatic DNA molecules and the polyacrylamide gel (74). Stellwagen (71) also observed that the absolute mobility of the bromphenol blue marker dye decreased linearly with increasing polyacrylamide gel concentration. This effect may be due to the affinity of polyacrylamide gels for aromatic compounds (74); the mobility of the marker dye was independent of gel concentration in agarose gels (71). The mobility of the marker dye was independent of electric field strength in either gel medium.

Retardation coefficients, K_R, were determined for DNA restriction fragments of different molecular weights (71). An approximately constant value of K_R was found for fragments larger than about 800 bp (71). For smaller fragments, $K_R \sim M^{0.2}$, which is a significantly different molecular weight dependence than observed in agarose gels ($K_R \sim M^{0.6}$, see above).

The values of K_R observed for the different fragments can be related to molecular dimensions by Eq. (1), if the molecular radius is chosen to the geometric mean radius (71). This means that the retardation coefficients are dependent on the mass of the DNA molecules (raised to the 1/3 power), even though these short DNA fragments are highly asymmetric in shape. Hence molecular mass, not molecular shape, appears to be the controlling factor in the polyacrylamide gel electrophoresis of DNA restriction fragments. It is possible that the DNA molecules act as "magic bullets" and deform the polyacrylamide gel during electrophoresis. Alternatively, the DNA molecules may interact specifically with the gel matrix during electrophoresis. Both of these processes would be expected to be mass dependent.

3.2.c Anomalous migration

When a series of linear DNA restriction fragments is electrophoresed in a polyacrylamide gel, the mobility of a significant number of fragments in the 300-500 bp range is found to be lower than the smooth curve describing the mobility of the rest of the fragments (71). Molecular weights of these anomalously migrating fragments, calculated from their electrophoretic mobility, would be 10-20% larger than their sequenced molecular weights (71). The anomalous mobilities are enhanced by increasing the polyacrylamide gel concentration (75), lowering the temperature of electrophoresis (71, 76), or by adding Mg^{++} to the electrophoresis buffer (76). Decreasing the polyacrylamide gel concentration (75), raising the temperature of electrophoresis (71, 76) or adding Na^{+} to the electrophoresis buffer (76) has the opposite

effect. The anomalously migrating fragments derived from plasmid pBR322 were found to originate from specific regions of the plasmid (71), suggesting that a conformational feature such as bending of the DNA helix is responsible for the anomalously slow migration. Dimerizing an anomalously migrating fragment further increases its anomalously slow migration (71).

The anomalously slow electrophoresis of some DNA fragments is particularly apparent for a kinetoplast DNA minicircle (75, 76); the molecular weight calculated from the electrophoretic mobility is 3-4 times larger than its sequenced molecular weight. This anomaly was attributed to a relatively sharp bend in the DNA rod (75). By making dimers of the kinetoplast fragment and cutting with different restriction enzymes, Wu and Crothers (77) were able to prepare a series of fragments of identical molecular weight, with the putative bend located in different positions with respect to the ends of the fragment. The mobilities of each of these fragments were compared and the bending locus identified, since fragments with the bend located near the end would be expected to have a higher mobility than fragments with the bend located near the center.

Hagerman (78) is undertaking a systematic study of the gel electrophoretic mobilities of a series of oligo(dA)-oligo(dT) sequences in order to determine the sequence dependence of the phase coherence between sequence periodicity and helical repeat.

Pseudo-cruciform formation has also been shown to be responsible for anomalously slow migration on polyacrylamide gels (79).

3.3 Denaturing gels

A large change in the electrophoretic mobility of a double-stranded DNA molecule in a polyacrylamide gel occurs when a portion of the molecule melts. Since melting is a cooperative phenomenon, the transition in electrophoretic mobility is abrupt if the electrophoresis takes place in a gel containing a gradient of denaturing solvent. The onset of melting is related to base sequence, since large DNA molecules melt in domains of different A-T content (80). Fischer, Lerman, and coworkers (81-83) have used this technique to separate DNA fragments differing by single base-pair substitutions (83, 84). Mutations affecting final gradient penetration lie within the first cooperatively melting sequence. If restriction fragments of the mutant DNA molecules are first separated in size by agarose gel electrophoresis, and then electrophoresed in the perpendicular direction in a polyacrylamide gel containing a denaturing gradient, two-dimensional separation of about one thousand fragments is possible (85). The effects of the size of the denatured region, the position of the denatured region in the molecule, and the molecular weight of the undenatured portion of the molecule on the mobility of the partially melted DNA molecule have also been investigated (86).

Another procedure has been developed to detect single-base substitutions in DNA fragments (87), based on ribonuclease cleavage of

single-base mismatches in RNA:DNA heteroduplexes (87). The digestion products are analyzed by electrophoresis in a denaturing polyacrylamide gel. This procedure has been used to detect single base mutations associated with human genetic diseases (87).

3.4 Sequencing gels

In the chemical DNA sequencing method, the DNA is end-labeled, partially cleaved at each of the four bases in four separate reactions, and the digestion products electrophoresed on denaturing polyacrylamide gels (88). Several hundred nucleotides can be separated using current electrophoresis procedures. From the nucleotide ladder produced, the base sequence of the DNA fragment can be read by inspection. Current applications of sequencing ladders, besides determining base sequence, involve the technique of "foot-printing". In this procedure, a ligand is bound to the DNA before the cleavage reaction. A modulated cutting pattern results; the absence of bands in the sequencing pattern indicates the location of the ligand (89, 90). This technique has also been used to measure the helical repeat of B-conformation DNA (91).

3.5 Protein-DNA interactions

A gel electrophoresis DNA-binding assay has been developed for the study of protein-DNA interactions (92-94). A major reduction in mobility occurs when DNA fragments are complexed with protein molecules. The gel matrix appears to stabilize the protein-DNA complex,

although the mechanism is not understood. A "caging" effect has been proposed: the gel matrix may form compartments that prevent the DNA from diffusing away from the protein (93). Alternatively, the protein and/or the DNA may interact with the gel matrix, or a discontinuity in ligand concentration may exist, similar to that observed with spermine (95).

3.6 Experimental procedures

The progressive increase in resistance which is sometimes observed in polyacrylamide gel electrophoresis was studied by Spencer (95-97). Changes in conductivity were attributed in part to the formation of a zone of altered salt concentration at the interface between the gel and the solution. A discontinuity in solvent concentration was observed, which migrated slowly into the gel. The effect was attributed to a change in transport numbers of the ions on two sides of an interface (96). There may also be changes in pH within the zone of altered concentration (97). Electrophoresis of tRNA in the presence of spermine resulted in a progressive decrease in current when running at constant voltage; the tRNA was contained in a sharp zone of precipitated material within the gel, at a slowly moving discontinuity in salt concentration. The region of the gel behind the moving boundary contained a much lower concentration of spermine than the electrophoresis buffer.

Righetti, et al. (53) measured the pore size of polyacrylamide gels, using polystyrene latex particles of known diameter. Highly diluted polyacrylamide gels (2% acrylamide, 2.2% crosslinker) were found to be

gluey and did not admit the latex particles, suggesting that the maximum pore size was smaller than 100 nm. Highly cross-linked gels containing 30% acrylamide were prepared using several crosslinking agents, including Bis. Below 25% Bis, no latex particles could enter the gel. At 25% Bis, the gel structure became porus (100 nm average diameter). The porosity increased rapidly up to 40% Bis, then increased at a slower rate up to 60% Bis. Gels containing more than 30% Bis were hydrophobic and exuded water. Cross-linking agents with very short chain lengths between the two terminal double bonds were needed to obtain highly porous gels.

Highly porous gels containing as little as 2.5% acrylamide were prepared in a different manner by adding 0.6% linear polyacrylamide to the gel matrix (98). The use of 0.4% diallyltartardiamide as a cross-linker gave clear gels without the loss of large pore size.

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Table I

Mobility at Zero Gel Concentration

Author	Reference	Type of DNA	$\mu \times 10^4$ $cm^2/Vsec.$
Olivera, et al	3	T2, T4 fragments	1.51*
Ross & Scruggs	30	calf thymus	1.85*
Johnson & Grossman	31	PM2	1.4
Serwer & Allen	28	linearized pBR322	3.1
Edmonson & Gray	26	λ-DNA restriction fragments	1.5-3.0
Stellwagen	6	0.5-12 kbp fragments	2.0†

*free solution, 0.1 M NaCl

†mobilities extrapolated to zero electric field strength

Table II

Birefringence relaxation time of DNA fragments in free solution and in agarose gels of different concentrations

BP	Free Solution (0.2 mM Tris Buffer)	0.2% Agarose Gel	0.3% Agarose Gel	0.5% Agarose Gel	1.0% Agarose Gel	1.5% Agarose Gel
622	τ = 52 ± 3 μsec L = 155 nm 74% of contour length	---	τ = 59 ± 4 μsec L = 163 nm 77% of contour length	τ = 59 ± 3 μsec L = 163 nm 77% of contour length	τ = 74 ± 5 μsec L = 185 nm 84% of contour length	106 ± 10 μsec L = 204 nm 100% of contour length
1426	305 ± 30 μsec L = 306 nm 64% of contour length	290 ± 90 μsec L = 300 nm 62% of contour length	850 ± 30 μsec* L = 452 nm 93% of contour length	1.02 ± 0.17 msec L = 483 nm 100% of contour length	1.12 ± 0.11 msec L = 496 nm 100% of contour length	1.29 ± 0.14 msec L = 527 nm 109% of contour length
2936	880 ± 50 μsec L = 578 nm 46% of contour length	2.2 ± 0.4 msec* L = 646 nm 65% of contour length	5.3 ± 0.5 msec L = 894 nm 88% of contour length	6.6 ± 1.1 msec L = 969 nm 100% of contour length	6.9 ± 1.2 msec L = 986 nm 100% of contour length	8.8 ± 1.1 msec L = 1088 nm 109% of contour length
Estimated median pore diameter	---	750 nm	520 nm	350 nm	190 nm	140 nm

*With 80 μsec orienting pulse; τ increases with increasing pulse length.

ABSTRACTS

THE FUNCTIONS OF A PROTEIN DATABASE DEVELOPED FROM COMPUTER-ANALYZED QUANTITATIVE TWO-DIMENSIONAL GELS. Stephen H. Blose, Laboratory for Cell Biochemistry, Protein Databases, Inc., 405 Oakwood Road, Huntington Station, New York 11746.

Protein databases are indispensible sources of dynamic protein information about living organisms. The information covers the spectrum of regulatory behavior of all the detected proteins in a cell; it tells about the proteins' genetics - hence the organism's genetics; it tells about the proteins' sub-cellular and tissue distribution; it tells which proteins are abnormal in various disease states; and protein databases will ultimately tell us the function(s) of the proteins in a cell or tissue. A unique property of protein databases is that they continue to evolve as more information is learned by an investigator and as more information is acquired from the scientific literature. The building blocks of protein databases are derived from computer-analyzed quantitative two-dimensional gel data obtained from many experiments, all referenced to a standard gel pattern. The foundation of the database provides protein information on:

*PROTEIN IDENTIFICATION
*SUBCELLULAR LOCATION OF PROTEINS
*POST-TRANSLATIONAL MODIFICATIONS
*PARTIAL AMINO ACID COMPOSITION
*RELATION OF PROTEIN PATTERNS TO CELL PHENOTYPE
*GENETIC EXPRESSION OF PROTEINS IN ORGANISMS
*PROTEIN PURIFICATION STRATEGIES
*ASSAY PROTEINS PRODUCED IN IN VITRO TRANSLATION SYSTEMS

The ultimate utilization of protein databases is to provide investigators with a better international medium to communicate results by providing readily understood quantitative information. When key proteins are identified, two-dimensional gels can rapidly purify the protein for antibody production or amino acid sequencing. This can be accomplished by using a thick preparative two-dimensional gel. The amount of protein recovered for each of the top 150-300 spots is in the pico-mole range. Protein databases function in other scientific fields by providing information on: protein purification, dynamic properties of protiens (factors that influence protein synthesis and secondary modification, expression of mutant proteins, rate of protein turn-over - the protein's half-life), functional protein complexes determined through chemical cross-linking and/or immunoprecipitation, protein translocation though cell compartments, protein binding properties (binding to DNA, lectins, and/or antibodies), cancer studies, growth factor studies, genetic engineering and breeding, genetic diseases, mutation and teratology studeis in animal and plant systems, clinical trials and diagnostic tests, drug and tocicology reseach; studies on single cells, cell lines, and cell senescence; quality control, and studies in the neurosciences. Effective use of databases to solve problems in each of these areas will be discussed.

GENETIC STUDY OF MARINE VERTEBRATE AND INVERTEBRATE POPULATIONS USING ULTRA-THIN ISOELECTRIC FOCUSING TECHNIQUES

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Two to eight different populations of two marine fish species and two marine crustacean species were studied using double isoelectric focusing on short (5 cm) ultrathin polyacrylamide gels (0.2 mm). Six to 35 different enzymes were studied on 50 to 300 individuals within each species. Linear and non-linear pH gradients were developed to obtain the best resolution of isoenzymes among species by mixing ampholytes (Pharmacia) of different pH ranges. Techniques were standardized for common reagents and showed species similarity. In contrast, pH gradients and isoenzyme patterns varied to a great extent among species, particularly for some enzymes.

DETECTION OF HEXOSAMINIDASE ISOZYMES BY SLAB GEL ISOELECTRIC FOCUSING (PAG-IEF)

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N-Acetyl-β-D-Hexosaminidase, a lysosomal enzyme, exists in two major forms: (1) β-hexosaminidase A (Hex A), a heat-labile heteropolymer ($\alpha\beta_2$) cleaves G_{M2} ganglioside, G_{A2} and hexosamine oligosaccharides; (2) β-hexosaminidase B (Hex B), a heat-stabile homopolymer ($\beta_2\beta_2$) degrades G_A, globoside and hexosamine oligosaccharides. Deficiency of Hex A results in Tay-Sach's disease; deficiency of Hex A and Hex B produces Sandhoff-Jatkewitz disease. Clinical manifestations of these diseases range from early death to late onset juvenile or adult forms and from normal clinical features to severe neurological deterioration.

The enzyme is separated by IEF using an ultra thin PAG with a pH 4 to 9. A gel consisting of 12% sucrose, 10% glycerol and 5% ampholyte with a T=5% and C=3% is cast on GelBond with a 2/100 inch spacer. The gel is prefocused for 1 hour with 5 mA constant current at 10°C on the LKB ultraphor with 0.5 M H_3PO_4 and 0.5 M NaOH as electrolytes. For optimal results, saturated LKB application pads of tissue culture homogenates, made in 0.05 M citrate buffer pH 4.3 are applied at the midline. Electrophoresis begins with 5 watts, 5 mA and 500 volts and is increased periodically to 1500 volts for a total run of 4 to 5 hours. The active bands for Hex A and Hex B are visualized by: (1) an overlay of freshly made substrate containing 10 mg napthol-AS-BI-N-Acetyl- β-D glucosaminide dissolved in 5 ml ethylene glycol monomethyl ether and mixed with 50 ml citrate buffer pH 4.5 and 10 mg garnet GBC salt. With incubation at 37°C for 60 to 90 minutes, the freed napthol couples with the fast garnet GBC producing a violet color. (2) an overlay of 0.1% 4 MUF N-acetyl-β-D-glucosamide in 0.1 M citrate buffer pH 4.5 incubated at 37°C for 30 minutes. Fluorescence is viewed with long wave UV light.

Hex A and Hex B each exist in at least 6 distinct bands with pI range of 4.5 to 5.4 (Hex A) and 7.2 to 7.9 (Hex B). We determined these banding patterns in homogenates of skin fibroblast cultures from patients with the various clinical forms of Tay-Sach, Sandhoff-Jatkewitz, AB variant as well as from obligate carriers and normal controls. In addition, the isozyme patterns have been determined in homogenates of liver, placenta and chorionic villi from normal individuals.

TITLE: NuFix™ Glyoxyl Agarose: A Versatile New Medium for Electrophoresis, Blotting and Chromatography

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ABSTRACT: The wide spectrum of glyoxyl agarose properties will be discussed:

1) Its controllable reactivity toward 1° and 2° amines
2) Its native fixation capability
3) Its potential for affinity electrophoresis
4) Its exceptional pre- and postimmobilization stability and
5) Its ability to be cast in any format

Glyoxyl agarose/PAG composite gels permit in situ immobilization of polypeptides after electrophoretic separation.

The application of glyoxyl agarose to blotting and chromatography will also be noted.

THE USE OF BIOTINYLATED HUMAN DNA PROBES FOR DETECTING RFLP'S

Human genomic DNA nick translated and labeled with bioten was used to hybridize and detect restriction fragment length polymorphisms, (RFLP'S) separated by electrophoresis and Southern blotted to nylon membranes. The detection system was a streptavidin-biotin-alkaline phosphatase complex which attached to the biotin ligand on the hybrids. The method was used to detect RFLP'S with a variety of single copy probes and numerous restriction endonucleases. The use of biotinylated probes for routine work was found to be more economical, faster and safer than the use of radioactive probes such as P32. RFLP'S could be detected with 2-5μg of human DNA with an electrophoresis, hybridization and staining procedure which could be accomplished in less than 2 days.

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[illegible] 1-[illegible] TRYPSIN (PI) PHENOTYPING BY ULTRATHIN LAYER ISOELECTRIC FOCUSING

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An effective economical method for the ultrathin layer isoelectric focusing of alpha 1-antitrypsin (Pi) was performed using Pharmalyte 4.2-4.9. This procedure, with an interwick distance of 9.5 cm, resolves the major Pi subtypes in 115 minutes on a 0.2 mm thick polyacrylamide gel. The use of a shorter interwick distance of 7.0 cm can reduce that time to 75 minutes. The S, M, M1, M2, M3 and some rare Pi variants are resolved and are easily detected by Coomassie blue or silver staining. For clinical diagnostic use, a rapid method suitable for the detection of the Z, or deficient, variant was also developed. The S, M, M1 and M2 are separated in 30 minutes across a narrow interwick distance of 5.0 cm, however M3 is not distinctly resolved.

NEW DEVELOPMENT IN HARDWARE AND SOFTWARE FOR 2D GEL ANALYSIS

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We have development of protein databases for rat, mouse, and yeast cells using a new hardware and software system for 2D gel analysis. The software, based on our earlier QUEST system, has been converted and substantially enhanced for use on a modern 32-bit workstation running under the UNIX operating system. Features of the new system essential to database development, and some examples from the database will be presented.

Protein databases must be contributed to and accessed by many users if they are to serve a useful function. The analysis of a major experiment (e.g. 10 gels of 1500 spots each) should not require more than overnight for the automatic processing procedures and not more than a few hours of interactive analysis by an operator. The Masscomp hardware, with its integral array and graphics processor, clearly allows this level of throughput. Automatic processing now includes automatic merging of all film exposures, accurate two-dimensional gaussian fitting of all spots, creation of composite standard patterns, and rapid matching and cross-matching.

New interactive features, such as rapid editing by spot quality, easy comparison of the gel image and synthetic spot image at any magnification, and the ability to refit spot clusters at any time allow user to verify and improve the quality of the automatic processing within a short time.

Each database is begun by building a core of information that describes the basic properties of the cells under study and their reproducibility under standard experimental conditions. Statistical analysis of multiple gels, multiple labelings, and multiple clones of cell lines are among the first experiments being analyzed. Some of the statistical controls and some new findings from the database will be presented.

AUTOMATED NUCLEIC ACID ELECTROPHORESIS AND HYBRIDIZATION ANALYSIS

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A computer controlled, fully automated system for Southern-type nucleic acid hybridization analysis has been designed and constructed. DNA digested with restriction endonucleases is loaded by the operator, into the sample wells of the gel, which is contained in a nine-fingered plastic frame. The operator then loads the P^{32} labelled probe(s) into the hybridization chamber. Instructions for all the steps in the automated process are specified by the operator's answers to questions which appear on the computer screen at the start of the experiment. Subsequent steps are performed automatically. The system performs horizontal, submarine electrophoresis. An adjustable endpoint detector terminates the electrophoresis. Automatic voltage/temperature feedback control maintains maximum allowable voltage while keeping temperature constant. Following electrophoresis a computer controlled robot arm moves the gel frame from station to station. The system then fixes the separated fragments to a solid phase matrix, denatures, neutralizes, washes, dries and detects the P^{32} according to the specifications preprogrammed by the operator. The results, printed out by the computer, give a plot of radioactivity versus distance from the origin for each of the nine simultaneous hybridizations.

Electrophoretic Seperation of High Density Lipoprotein Fractions.

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ABSTRACT

A clinical laboratory method is presented for use in the determination of the relative quantities of the two major high density (alpha fraction) lipoprotein fractions present in human serum. The method described was developed in this laboratory to help in the evaluation of coronary artery disease risk factors for patients. Methods such as ultracentrifugation can be used to determine HDL_2/HDL_3 ratios, but they require special equipment and great skill. This procedure involves removing the low density lipoproteins from the serum sample, then fractionating the remaining high density lipoproteins by electrophoresis on special gelatinized cellulose acetate strips. Visualization is done by staining with a fat stain, or by using a cholesterol reagent. The ratio is obtained by scanning the wet strip on a densitometer.

PHYSICAL CHARACTERIZATION OF TURNIP CRINKLE AND PELARGONIUM FLOWERBREAK VIRUSES BY AGAROSE GEL ELECTROPHORESIS: APPLICABILITY OF POLYSTYRENE SIZE STANDARDS AND OF MOVING BOUNDARY ELECTROPHORESIS BUFFER SYSTEMS.

E. Gombocz, D. Tietz, S. Hurtt and A. Chrambach, NIH, Bethesda MD 20892 and * USDA, Beltsville MD 20705.*

Turnip Crinkle and Pelargonium Flowerbreak Viruses were physically characterized by quantitative agarose gel electrophoresis at pH 6.5, 0.03 M ionic strength, 2 ^{o}C, 10 to 50 mM CHAPS, using N-β-hydroxyethylmorpholinium-ACES buffer either continuously in all phases (2 V/cm) or as the gel buffer within a discontinuous buffer system (1.2 mA/cm^2). Agarose (Iso-Gel, LKB) at concentrations ranging from 0.1 to 2.3% was applied in standard thermostated gel tube apparatus. Viruses were reacted with ethidium bromide prior to electrophoresis and detected by the bound fluorescence. Polystyrene size standards (Polybead Microspheres, Polysciences, Warrington PA) ranging in nominal radius from 28.5 to 75 nm were visible zones.

Stacking of both the viruses and the polystyrene standards in 0.4% agarose required a) trailing and leading ion net mobilities relative to Na of 0.059 and 0.273 respectively; b) CHAPS concentrations of 50 mM in all phases; c) current densities not exceding 1.2 mA/cm^2.

Non-linear Ferguson plots (log(mobility) or log(R_f) vs. agarose concentration) were obtained for viruses and standards. The slopes of the lines connecting points on the curves with the curve intercepts on the mobility axis, K_R', were determined. Coefficients obtained by curve-fitting for the standards were applied to the appropriate functions to compute particle radii of the viruses on the basis of K_R' values.

Immobilized pH gradients for one and two-dimensional separations. Applications.

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A high degree of resolution and reproducibility is achieved with isoelectric focusing in narrow (< 0.2 pH units / cm) and ultra-narrow (0.05 - 0.01 pH units / cm) immobilized pH gradients (IPG). Human plasma proteins (e.g. Gc, Tf, Pi) analyzed for genetic variability are distinctly resolved and located for accurate subtyping (1,2,3). By using narrow IPG in the first dimension closely related proteins, which tend to be clustered into small areas of the 2D map, are better resolved (4). Proteins from yeast and legumes are focused in broad IPG (pH 4-10) in the presence or absence of urea, detergents or carrier ampholytes and applied to the second dimension. The resulting 2D maps differ essentially reflecting the findings obtained with IEF in three different systems (IEF, IPG, IPG-CA). In comparitive experiments, two identical IEF strips were applied to a horizontal and a vertical SDS pore gradient gel. The 2D maps show no significant differences with respect to resolution, spot size and distribution, altnough the horizontal system is easier to perform than the vertical system. The equilibrated IEF gel strip on plastic support is simply loaded on the surface of the horizontal SDS pore gradient gel (500 µm thick on Gel Bond PAG film). No agarose overlay or embedding gels are needed (5).

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A NEW PROGNOSTIC INDICATOR IN ACUTE LYMPHOBLASTIC LEUKEMIA DETECTED BY TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS (2-D PAGE).

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In previous studies we have utilized 2-D PAGE to detect polypeptide markers in acute lymphoblastic leukemia (ALL). Sixteen polypeptides were detected that, as a group, distinguished between CALLA positive non-T, non-B; CALLA negative non-T, non-B; B cell and T-Cell ALL. Over the past four years 72 patients with ALL have been studied. One polypeptide, L3 (M W 29,000) was observed in all 53 CALLA positive and nine CALLA negative non-T , non-B ALL patients, but was not detected in 6 T-cell and 4 B-cell ALL cases nor in 15 patients with AML. In contrast to other markers, substantial variability in the intensity of L3 was observed between patients. The variable intensity of L3 led us to search for clinical or pathological correlates. A strong correlation was observed between the intensity of L3 and clinical outcome. Of 18 patients with a faint L3, 10 have relapsed; ;whereas 3/19 with a moderately intense and 1/15 with a heavy L3 spot have relapsed. Of the patients who have been followed in our study for two years or longer, 9/10 with a faint L3 spot have relapsed; whereas none of 10 patients with an intense spot have relapsed. There was no correlation between the intensity of L3 and other established prognostic indicators. Therefore, the detailed analysis of the polypeptide constituents of leukemic cells, as made possible by 2-D PAGE, has revealed a previously undetected polypeptide, the deficiency of which is associated with an unfavorable outcome in non-T, non-B ALL.

Mechanistic Studies of Silver Staining

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Much controversy exists as to the mechanism of silver stains and to the importance of several variables. We are attempting to probe the mechanism of the stain or stains and to find answers to questions such as: Does the Ag+ bind specifically to the protein and if so to what groups in the protein? Is the binding ratio characteristic of the protein? Does the binding step determine the overall sensitivity of the stain, and if so how can we adjust conditions (e.g. pH, ionic strength, temperature) to maximize the binding? Why is the Ag+ reduced mainly in the vicinity of the protein and how can we minimize non-specific (e.g. background) reduction?

We have carried out preliminary silver-protein binding studies using a variety of measurement techniques. Bovine serum albumin was titrated with 10^{-3} mol/L $AgNO_3$ using a Ag ion specific electrode to monitor Ag concentrations. Neutron activation analysis has been used to map Ag concentrations in polyacrylamide slab gels that were stained with either a Merril-type silver stain or with an ammoniacal silver stain. The feasibility of using ^{109}Ag NMR to study silver-protein binding sites has been investigated. Results of these studies will be presented.

Studies on Catechol-O-Methyltransferase in Rat Brain Using Two-Dimensional Gel Electrophoresis

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The presence of catechol-O-methyltransferase (EC 2.1.1.6) in the rat brain was studied using a combination of two-dimensional gel electrophoresis, protein blotting and a specific antiserum. Two major immunoreactive proteins were identified - one with a molecular weight of 23 kDa and an isoelectric point of 5.2, the other of molecular weight 25 kDa and isoelectric point of 5.1. In addition, multiple lower molecular weight immunoreactive proteins, possibly corresponding to breakdown products of the enzyme, were also detected. The 23 kDa form of catechol-O-methyltransferase, which is probably the soluble form of the enzyme, is a major protein visible on silver-stained two-dimensional gels of rat brain. In contrast, the other proteins recognized by the antiserum were not detected by the silver stain.

These results demonstrate, using two-dimensional gel electrophoresis, that at least two distinct forms of catechol-O-methyltransferase are present in rat brain. In addition, since one of these proteins is stained by silver, these results also serve to identify another protein visible on two-dimensional electrophoretograms of rat brain.

IMMOBILINE ELECTROPHORESIS: pI VALUES FOR PROTEINS OF LDH-4 AND -5 FROM CONTROL AND NITROSOMETHYLUREA-TRANSFORMED NEOPLASTIC CELL LINES.

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Agarose and PAG electrophoresis of LDH-4 and LDH-5 from a control cell line established from rat hepatocytes, TRL, and its nitrosomethylurea-transformed neoplastic cell line, NMU-3, shows that the proportions of the two isozymes differ in the two cell lines. In TRL cells, LDH-4 is three to four times more active than LDH-5, whereas in NMU-3 cells the reverse is observed. Following PAG electrophoresis, three faint protein bands are detected with the silver stain method of Wray and Wray (Anal. Biochem. 118, 197, 1981). Two proteins are parallel to the two LDH isozymes but a third is not and does not react as LDH. First attempts to identify the pI values of the two isozymes were carried out with the isoelectric focussing (IEF) method. LDH-4 separated into 5-6 proteins which migrated below pH 7; LDH-5 migrated to the alkaline region of the gel, to at least pH 8.8, but could not be better-defined in this system. With the application of Immobiline electrophoresis (IE) the results to date are as follows: Eight proteins are distributed between LDH-4 and -5. LDH-4 is characterized by six proteins with pI values ranging between pH 5.3 and 6.8. LDH-5 is characterized by two proteins with pI values above pH 9.0. The results with IE confirm the wide pH difference between the pI values of the proteins associated with each of the two LDH isozymes. The two proteins identified thus far with LDH-5 appear to have pI values above pH 9. These values are among the highest reported thus far, only cytochrome c being above pH 10.

PARAMETRIC VERSUS NON-PARAMETRIC GEL IMAGE ANALYSIS

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ABSTRACT

Two different sets of methods for spot detection and quantification in digital images generated by two-dimensional gel electrophoresis have been described in the literature: (1) parametric methods that operate on the basis of a Gaussian spot model, and (2) non-parametric methods that do not rely on any assumptions concerning the shape of spots. There are no data available that would allow to judge the relative advantages and disadvantages of either approach to gel image analysis.

Therefore, we performed a set of experiments involving computer simulations in order to compare the accuracy of quantification of two typical methods, subsequently referred to as A and B from classes (1) and (2) respectively, implemented on a general purpose image analysis system. The simulated images consisted of a number of Gaussian spots with differing degrees of overlap to which random noise with Gaussian distribution density was superimposed at different signal to noise ratios. Only isolated spots were included in the analysis.

Preliminary results indicate that the differences in relative errors and variability of the estimates between methods A and B depend on spot intensity in case of a non-linear OD-dpm relationship. The coefficient of variation (cv) of method B estimates tends to be lower than the cv obtained with method A. This was particularly well noticed for OD ranges for which the slope of the OD-dpm curve was steepest. This is due to the fact that errors committed during an imperfect Gaussian fitting process in the OD domain are amplified in the dpm domain through the non-linear OD-dpm conversion. In addition to considerations regarding the amount of data reduction achieved by methods A or B, computational efforts involved, ability to regenerate synthetic gel images for visual inspection, etc., these results are of great importance in selecting algorithms for an optimum gel image analysis system.

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2,5-DIPHENYLOXAZOLE (PPO) IN DIMETHYL SULFOXIDE (DMSO) REGENERATION FOR RE-USE IN FLUOROGRAPHY

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ABSTRACT

At present, impregnation of the scintillator PPO dissolved in DMSO into two-dimensional electrophoresis gels for fluorography, as described by Bonner and Laskey in 1974, remains one of the most sensitive and widely used methods for detecting and quantifying radioactivity in tritium- and ^{14}C-labeled proteins. The relatively high cost of this scintillator often necessitates the reuse of PPO from partially depleted solutions. In an attempt to recover PPO from the DMSO solvent for further use, researchers have precipitated PPO with water, filtered, and dried it. We have developed a simple fluorescence assay for measuring the amount of PPO remaining in a partially depleted solution of PPO in DMSO. A calculated amount of PPO can then be added back to the partially depleted solution to restore the initial PPO concentration. Thus, PPO solutions can be regenerated and used many times before being precipitated due to the accumulation of water.

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ANALYSIS OF MICRO-VARIATIONS IN THE RATE OF SYNTHESIS OF INDIVIDUAL POLYPEPTIDES ISOLATED FROM CULTURES OF THE RAT HEPATOMA CELL LINE H4-II-E AS MEASURED BY QUANTITATIVE TWO-DIMENSIONAL GEL ELECTROPHORESIS.

by Mark J. Miller and Snorri S. Thorgeirsson, Laboratory of Experimental Carcinogenesis, National Cancer Institute, Bethesda, MD. 20892.

Living cells maintain themselves in a delicate metabolic balance, adjusting to changes in their external and internal environments through a wide range of biological processes. One of these processes is gene expression, which manifests itself as protein synthesis. Two-dimensional polyacrylamide gel electrophoresis has been used in numerous studies to detect modulation in the rate of protein synthesis as cells progress through the cell cycle, are subjected to heat shock, or undergo neoplastic transformation. Many of these changes are quantitative, reflecting a change in the rate of synthesis rather than a turning on or off of synthesis. To date, we know of no study aimed at determining the baseline level of variability in steady state cell cultures.

Using a computerized analysis system capable of detecting changes in the rate of protein synthesis of as little 30%, we have examined the rate of synthesis of over 900 polypeptides in two independent subclones of the rat hepatoma cell line, H4-II-E (Reuber Cells). Cells from both subclones were grown in the same media lots, and all experiments were done on log phase cells at approximately the same cell concentration. Only 7 proteins were detected that were <u>qualitatively</u> different between the two subclones; i.e. a spot was consistently present in one subclone, but undetectable in the other. Some of these spots may be modification products of one another. On the other hand, 9% of the spots showed statistically significant <u>quantitative</u> differences when compared, in triplicate, between the two subclones. Duplicate extractions of the same culture indicate these differences are not artifacts of sample preparation or electrophoresis. Multiple experiments indicated that, although there was always a significant degree of heterogeneity in the quantitative expression of proteins between the two subclones, these differences involved different sets of proteins from experiment to experiment. A time course experiment, in which samples were taken and analyzed every week for 5 weeks indicated that the expression of certain polypeptides can vary significantly during the life of the culture. These experiments indicate that the differences observed represent a dynamic within the cell culture and not, generally, a difference between the two subclones. The nature and cause of this variation is under investigation.

β-Hemoglobin Polymorphism in White-Tailed Deer: Suitability and Application to Wildlife Law Enforcement.

Rodger Morrison and Morris G. Brown

The β-hemoglobin types of over 250 Virginia white-tailed deer (Odocilus virginianus) have been determined by cellulose acetate electrophoresis. Nine different genotypes have been observed in the sample from the deer population of the Skyline Management Area, in Jackson County, Alabama. The homozygous type III was found to be the most common (86.6%). Gene frequencies for the five Hb_{β} alleles are as follows: II (0.028), III (0.921), IV (0.014), V (0.028), VII (0.008). All genotypes other than the homozygous type III were confirmed by isoelectric focusing in thin acrylamide gels, pH 5-7.

In forensics, species identification of deer blood has been the main objective, particularly in areas where closely related species are found. This represents a step towards intraspecies individualization in white-tailed deer. β-hemoglobin appears to be a stable marker. Both cellulose acetate electrophoresis and isoelectric focusing were found to be reliable procedures for the determination of β-hemoglobin types. The method has been used in a wildlife law enforcement case involving a deer killed in the Skyline Management Area.

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PREPARATION OF URINE AND SERUM SAMPLES WITH CENTRIFUGAL MICROCONCENTRATORS AND TOTAL PROTEIN ANALYSIS BY ULTRAVIOLET-DIFFERENCE SPECTROSCOPY FOR TWO-DIMENSIONAL ELECTROPHORETIC ANALYSIS

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Two-dimensional electrophoresis (2DE) requires concentrated and desalted urine samples or diluted and desalted serum. We have developed procedures for the ultrafiltration and diafiltration of preserved (sodium azide, sodium carbonate, and aprotinin) urine and serum with centrifugal microconcentrators fitted with 10,000-dalton cutoff filters. Parallel processing with a 24-position rotor allows up to 20 mL of each urine sample to be ultrafiltered in 12-14 centrifugation steps, which achieves approximately a 200-fold concentration, and then diafiltered with water in 2 steps. This processing is completed in about 2.5 days. The filtration rate for urine averaged 1.3 mL/centrifugation (6,000 x g for 30 min) and only slightly decreased with each centrifugation. Retention of ^{125}I-*beta*-2-microglobulin (M_r=11,800 daltons) on the filters was virtually 100%, and <10,000-dalton proteins were absent on calibrated and silver-stained 2DE gels of urine or serum, confirming the sharp M_r cutoff. After the diafiltration step, the retentates of 24 human urine samples were analyzed for total protein by ultraviolet-difference spectroscopy, Coomassie dye binding, bicinchoninic acid (BCA), and Lowry methods. The UV-difference method compared most favorably with the Lowry method, was the easiest to implement, and required no additional reagents other than protein calibrators and water diluent. Urinary 2DE protein patterns from normal samples prepared as above showed at least one major group of spots, which is absent from samples prepared with gel exclusion methods and which is removed by cellulose nitrate filtration. The microconcentrator method is suitable for large studies in which reproducibility over long periods is important.

TITLE: Effects of Borate on Agarose Gel Structure

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ABSTRACT: Borate buffers change the sieving properties of agarose gels when compared to non-borate buffers. In both agarose (SeaKem® LE) and low-melt agarose (SeaPlaque®, NuSieve®), substitution of borate for acetate increases the apparent sieving of DNA fragments (72bp to 3000bp, ca. 40,000 kD to 2MD). The sieving increase due to borate is most pronounced if the borate is present during casting of the gel.

Borate also alters agarose viscosity, gel strength, and gelling and melting temperatures. Using an analysis similar to that of Serwer et al (Electrophoresis 4, 232-236, 1983), it appears that borate may bind to agarose in such a way as to reduce the packing density of helices during gel formation.

Results of an Interlaboratory Round Robin using Isoelectric Focusing to Assess Reproducibility of Rehydratable Gels

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During the past year, the National Bureau of Standards conducted an interlaboratory comparison study to test the effectiveness of rehydratable gels in obtaining consistent patterns with standard protein preparations. Each of five laboratories was provided with a package containing standard protein samples, rehydration media with instructions, techniques for silver staining and ampholines. Laboratories were allowed to run gels by their existing methods or whatever method they felt would give the best results.

In comparing laboratory results it was noted that there were distinguishable variations in the protein band patterns of the same protein due to several factors affecting the electrophoretic run. Factors identified include: 1) dependence on electrode separation distance, voltage and time of run; 2) nature of the electrolyte solutions; 3) quantity of sample applied and method of application; 4) type of apparatus used; and 5) relative humidity at the time of the run. Of the above, it appears that the type of apparatus used was a major factor in producing differences. The second most significant difference appeared to be the electrode separation distance.

A second round robin needs to be run taking into account the above factors. In addition, the general running conditions will be specified, if possible, and anolyte and catholyte solutions will be provided along with suggestions for sample application.

ELEVATED LEVELS OF p21 ras DETECTED IN BLAST CELLS FROM LEUKEMIC PATIENTS.

Atul Sahai and Samir M. Hanash. Department of Pediatrics, University of Michigan Medical School, Ann Arbor, Michigan.

Activation of the cellular oncogene ras has been implicated in many types of human malignancies including hematopoietic tumors. However, most studies of ras oncogene expression have examined either the ability of DNA derived from tumor cells to transfect NIH 3T3 cells or the level of mRNA in the cells, and few have examined the protein products of the celllular oncogenes. In the present study: 1) We have analyzed the polypeptide patterns of lymphocytes obtained from the peripheral blood of healthy individuals and of blasts from leukemic patients using two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). Protein patterns were visualized by silver staining and fluorography. 2) We report the expression of the p21 ras product in blast cells from various leukemic patients. Lymphocytes from healthy donors and from leukemic patients were simultaneously labelled with ^{35}S-methionine (200 uci/ml) for overnight and cell lysates were immunoprecipitated using monoclonal antibody Ab-1 (Oncogene Sciences, New York) that detects C-Ha-ras, C-Ki-ras and N-ras forms of the protein. Untransformed NIH-3T3 cells and 3T3 cells transformed by viral Harvey ras were used as negative and positive controls, respectively. 2-D PAGE of immunoprecipitates detected elevated levels of p21 ras in some of the leukemic patients. The amount of p21 ras observed was below the detection threshold of silver staining techinique as well as fluorography. Under the conditions used in our study while it apperas that the p21 polypeptide product of ras oncogene is not detectable in leukemic cells by either silver staining or fluorography, several other polypeptide alterations were observed. The significance of these alterations remain to be determined.

INTERMEDIATE RANGE MOLECULAR WEIGHT MARKERS FOR SDS GELS

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Molecular weight markers for SDS and 2-D electrophoretic gels are essential for intra and inter-laboratory comparisons of protein patterns. The molecular weight markers used presently with Coomassie blue stains are not suited for ultrasensitive silver stains because of microheterogeneity from contaminating proteins, proteases, and oxidation of sulfhydryls. The test criteria applied in selecting ideal markers are band thickness, pattern stability, purity, color, linearity, and reproducibility. Four markers, having apparent molecular weights of 81,000, 40,500, 29,000 and 17,500 daltons are recommended as intermediate range molecular weight markers for SDS gels.

The Effect of Calcium Ion Concentration on the Electrophoretic Mobility of Two Cultured Human Embryonic Kidney Cell Populations

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A very low ionic strength buffer is used for the Continuous Flow Electrophoretic Separator (CFES) operated by NASA and McDonnell-Douglas Astronautics Co. The absence of sodium and low levels of other ions in this buffer provides an opportunity to study the effects of particular ions on the Electrophoretic Mobility (EPM) of cells. Human Embryonic Kidney (HEK) cells were used in this experiment because they are used in an ongoing NASA project to electrophoretically purify urokinase producing HEK cells and because they are electrokinetically heterogeneous. A Pen Kem System 3000 Automated Electrokinetic Analyser was used to measure the EPM of two cultures of HEK cells derived from different sources. Calcium ion concentration was varied from the standard CFES concentration of 0.0274 mM to 27.4 mM. As expected on the basis of published results and theoretical considerations, increased calcium ion concentration led to decreased EPM, with overall population heterogeneity remaining about the same. The lowest mobility subpopulations of cells had near zero or positive mobility at the highest calcium ion concentrations.

This work was supported by the U.S. National Aeronautics and Space Administration.

Advantages of Focusing Carbamylation Trains in a Flat-bed System

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Carbamylation trains are usually visualized in the second dimension of two-dimensional electrophoresis systems. This can be a lengthy and labor-intensive process. Our need to compare the carbamylation patterns of many different proteins requires a quicker method, one which would allow simultaneous running and viewing of several samples.

Our method is a polyacrylamide flat-bed isoelectric focusing gel of the same recipe as one might use in the first dimension of a 2-D system. This gives several advantages over running rods:

- separate lanes on one flat gel are easier to compare than separate rods which may vary in length
- the sample application site is not limited to the anode or cathode
- extended pH gradient due to less cathodic drift
- flat surface allows use of a microelectrode for apparent pH determination
- easier to handle than rods

This system allows a rapid comparison of separations occurring during the isoelectric focusing dimension of the 2-D system.

This poster will outline our flat-bed isoelectric focusing method and show the results obtained.

TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELETROPHORESIS OF LYMPHOCYTE PROTEINS WITH IMMOBILIZED pH GRADIENTS IN THE FIRST DIMENSION. John R. Strahler, Samir M. Hanash, Luke Somerlot. Department of Pediatrics, University of Michigan Medical School, Ann Arbor, Michigan and Angelika Gorg, Lehrstuhl fur Allegemeine Levensmittel Technologic der Technischen Universitat Munchen, Germany.

We have investigated the use of isoelectric focusing in immobilized pH gradient (IPG) gels as the first dimension in two-dimensional polyacrylamide gel electrophoresis of lymphocyte proteins. IPG gels were compared in the range between pH4 and 10. Washed, dried and rehydrated IPG gels were run in the presence of 8M urea, NP-40, dithiothreitol, and the presence or absence of free carrier ampholytes. Lymphocyte proteins, solubilized with 9M urea, 2% NP-40 and carrier ampholytes, were applied to individual IPG gel strips. Gels were focused for 1 hr (1 w constant power) until the sample had entered the gel and then for 5 hr at 5 w constant power (3,000 v maximum). The strips were then equilibrated for varying times at pH 6.8 or 8.6 with urea, SDS and mercaptoethanol, and applied to the second dimension SDS polyacrylamide gradient gel. Polypeptides were visualized by silver staining. The majority of polypeptides visualized have acidic pI's (pH range 4-6). Of particular interest however were the polypeptides seen in the alkaline region. Polypeptides with pI's in the range 7 to 9 do not focus well or are not seen in our 2-D gels using conventional isoelectric focusing with carrier ampholytes. The inclusion of the non-ionic detergent NP-40 in the IPG gel disturbed the focusing in the alkaline range but the effect was minimized by using shorter focusing times. Carrier ampholytes in the IPG gel increased the net transport of protein into the gel, nevertheless there was significant precipitation of sample at the site of application. The use of IPG gels in the pH range 7 to 10 and containing carrier ampholytes, urea and NP-40 will make it possible to analyze the basic proteins of lymphocytes and other cell types.

PHYSICAL CHARACTERIZATION OF PARTICLES AND GEL FIBERS ON THE BASIS OF NON-LINEAR FERGUSON PLOTS IN AGAROSE GEL ELECTROPHORESIS. *D. Tietz, E. Gombocz and A. Chrambach, NIH, Bethesda MD 20892.*

The non-linear Ferguson plots observed in the quantitative agarose gel electrophoresis of viruses (Serwer et al., Gombocz et al.), other subcellular particles (Gottlieb et al.) and polystyrene microspheres (Gombocz et al.) call for an evaluation of particle radii and gel fiber properties on the basis of a retardation coefficient variable with gel concentration (Tietz et al.). Mobility data of proteins, viruses and polystyrene particles were analyzed by computer simulations, using program MLAB (DCRT, NIH). The applied mathematical model uses functions derived from the Ogston theory. Parameters which originally were constants are replaced by variables dependent on gel concentration and particle size. Correspondingly, the retardation coefficient K_R (which is constant for each particle when its Ferguson plot is linear) is replaced by a variable K_R' defined for each gel concentration by the slope of the line connecting a point on the curve with the curve intercept on the mobility axis.

Computer modeling 1) accounts for the apparent paradox of different fiber radii derived from the agarose gel electrophoresis of proteins and viruses, by demonstrating a continuous variation of effective agarose fiber radius on gel concentration; 2) demonstrates a dependence of effective agarose fiber radius on particle size; 3) indicates compressibility of polystyrene microspheres at high agarose concentrations; 4) allows one to compute particle radii of unknowns in the range of 15 to 80 nm, using commercial polystyrene microspheres as size standards.

PREPARATIVE AND ANALYTICAL IMMOBILINE ELECTROFOCUSING OF CLASS III HEPATIC ALCOHOL DEHYDROGENASE. K.H. Valkonen and D. Goldman (Unit of Genetic Studies, Laboratory of Clinical Studies, NIAAA, Bethesda, Maryland 20892).

We have resolved human and rat hepatic Class III alcohol dehydrogenases (ADH) by analytical and preparative immobiline electrofocusing. Separations were carried out in narrow (pH 5.3-6.9) immobilized gradients. Both in human and in rat there were three bands which stained with cinnamol but not with ethanol and which were insensitive to the inhibitory effect of pyrazole. The isolelectric points of the Class III ADH bands, determined in analytical gels by using commercial isoelectric markers, were 6.3, 5.9 and 5.6. The three Class III ADH bands from human liver showed the same isoelectric points in preparative immobiline gradients as in analytical gels. The enzyme electroeluted from each Class III ADH band was active with the long chain alcohol cinnamol (7.5 mM), was not saturated even by 2 M ethanol, and was not inhibited by 12 mM 4-methylpyrazole. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed that the eluted Class III ADH bands consisted primarily of a single 43 kd molecular species corresponding to Class III ADH, and lesser amounts of a 65 kd contaminant. Immobiline isoelectrofocusing thus provides a sensitive analytic method for the detection of charge variants and a rapid preparative separation method for Class III ADH.

Sequential Analysis of Chemically Induced Hepatoma Development Using Two-Dimensional Electrophoresis (2D-PAGE)

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It is becoming increasingly apparent that cancer development in many tissues or organs is a stepwise process involving altered cell populations at many steps. The liver offers a very attractive model for the study of carcinogenesis since the process of hepatocarcinogenesis appears to follow a "programmed" series of distinctive cellular and tissue changes which ultimately result in the formation of hepatocellular carcinomas. It seems likely, however, that the acquisition of the neoplastic phenotype would bring about both qualitative and quantitative changes in cellular functions that are distinctly different from those observed under normal and/or preneoplastic conditions. Utilizing a number of experimental carcinogenesis protocols our laboratory has undertaken the sequential analysis of polypeptide changes during hepatocarcinogenesis in the rat using 2D-PAGE. Analysis of 1000-1200 silver stained cytosolic and membrane-associated polypeptides under both equilibrium and nonequilibrium (NEPHGEL) conditions revealed four qualitative and numerous quantitative polypeptide differences between normal liver and preneoplastic (PN) and neoplastic (N) tissues. One cytosolic (pI 6.8/57 kDa) and three membrane-associated polypeptides (6.25/41, 6.75/24, and 6.05/21) were expressed in both PN and N tissues but not in normal liver. In addition roughly 4-10% of the polypeptides were undergoing quantitative changes of at least four-fold during these stages of hepatocarcinogenesis. These data suggest that gene expression is not extensively altered during early stages of heptocarcinogenesis.

FOUNDATION EXPERIMENTS FOR BUILDING A PROTEIN DATABASE. Virginia P. Wray, Protein Databases, Inc, 405 Oakwood Road, Huntington Station, New York 11746.

Building the foundation of a protein database that organize the protein information can be initiated by conducting several initial experiments and examining the results on computer-analyzed two-dimensional gels. The initial experiments include:

1. Determine the optimal conditions to radiolabel the system's proteins to steady-state with various aminoacids (i.e., [^{35}S]-methionine, [^{3}H]-leucine, [^{3}H]-proline).
2. Map all the protein "players" in the system on several combinations of isoelectric focusing gels and second-dimension acrylamide gels.
3. Quantitate the amino acid composition of all the proteins in the system to establish specific "signatures" for each protein.
4. Determine secondary post-translational modifications of the proteins as part of their signature and regulatory behavior (phosphorylation, glycosalation, methylation, fatty acid acylation, etc...) where appropriate.
5. Pulse-chase studies to determine the turnover (half-life) of all resolved proteins.
6. Other suggested experiments:
   - A. Identify key known proteins and enzymes by mixing in purified proteins and by immunoprecipitation to establish key landmarks.
   - B. Compare and Quantitate:
     - >Protein changes after various experimental manipulations such as: drug dose-response, growth, development, etc.
     - >Protein differences produced by genetic manipulations (map phenotypic changes).
     - >Proteins derived from cell fractionation studies to determine the protein's subcellular location.
     - >Proteins obtained from different tissues of an organism to establish tissue specific protein markers.

The assembled data constitutes a protein fingerprint for an experimental cell or tissue model. This type of database continues to evolve as more information becomes available from experimentation and the scientific literature. Protein databases can be utilized in many areas of cell biology and medicine to accelerate accumulation of new research data.

MECHANISM OF NICKEL AND SILVER VISUALIZATION of PROTEIN ELECTROPHOREGRAMS

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The so-called metal stains have found increasing use in protein electrophoresis, particularly where improved sensitivity is desired.

Such visualization systems are examples of what is called physical development in the photographic field, and electroless plating in the metal plating industry. The determination of the sensitivity and amplification factors of these systems is based on well-known principles of heterogeneous catalysis.

A description of the chemistry, nucleation, and amplification processes involved in nickel and silver visualization systems will be presented, together with calculations describing their ultimate sensitivity.

COMPARISON OF 2-DIMENSIONAL GEL ELECTROPHORETIC SEPARATIONS USING SOFT LASER SCANNING DENSITOMETRY WITH COMPUTER COLOR GRAPHICS

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Two autoradiograms of two-dimensional slab gels (5"x5") showing electrophoretic separations of normal and cancerous mouse skin fibroblasts were individually scanned using a computer-aided soft laser scanning densitometer. The contour of the abnormal state (red color) was overlaid electronically on that of the normal (green color) sample on the screen for direct comparison. In this way the user was enabled to detect the appearance (red) or disappearance (green) of protein spots in the test sample at a glance. The quantitative differences were shown in the computer printout in the form of tabulated numerical values such as, spot number, X-Y position, peak height and amount of protein. In the event the images could not be superimposed properly due to gel distortion the user was enabled to manipulated the data in the test sample while the control data remained on the screen fixed. Flexible superimposition was accomplished using the following functions: (1) slide: right, left, up or down; (2) expand - compress: in the X or Y axes and (3) rotate: clockwise or counterclockwise. In general, the system was found easy to use and it facilitated quantitative and qualitative comparison of complex protein spot patterns revealed on two-dimensional gel electrophoregrams in a time-saving manner.

MULTILANE STRIP-CHART PRINTING PROGRAM FOR DNA SEQUENCE READING

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A computer system has been developed which allows superimposition of 4 lanes (A, G, C & T bases) on chart paper, in different colors, for determining the position of bases shown on X-ray film. The system when used in accord with a high resolution- high sensistivity soft laser scanner offers the following distinct advantages: (1) Ensures accurate DNA sequence reading, since determining the position of a base among different lanes, becomes an easier and more precise process when bases are identified as "peaks" in densitometric tracings rather than as "bands" in electrophoretic separations on autoradiograms. (2) Electronic expansion of the four-lane graphics (A, G, C & T) provides clear and easy reading of bases and especially when graph paper is used for computer printout. The vertical lines on the paper serve as reference points that help to determine the relative base positions more accurately. In addition to this, DNA sequence reading is further facilitated by printing of each of the four tracings in different color. This feature provides distinct identification of the individual lanes, facilitating thus reading of base sequence.

The system described for DNA sequence reading is a powerful analytical tool which eliminates ambiguities as well as tedious and time-consuming work inherent to manual methods.

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